Kill or Cure:
Radiation Damage in Cryocooled Macromolecular crystals.

APS, July 2004

Elspeth Garman, Oxford
elspeth@biop.ox.ac.uk

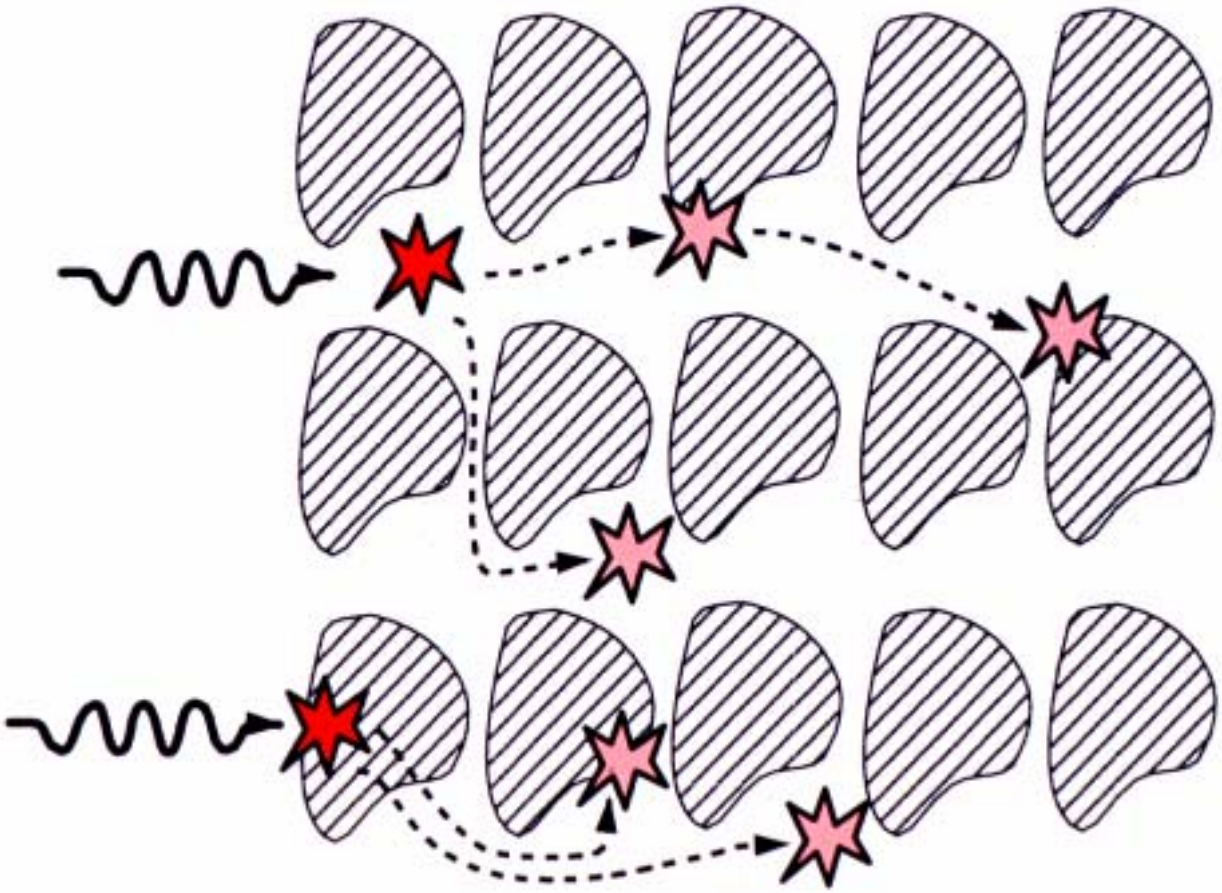
The Plan:

- **Radiation damage: what is it?**
- Maximum theoretical tolerable dose.
- Why does it matter?
- Can we control it?
- Or even use it?

Radiation Damage

Primary:

Secondary:



PRIMARY; inevitable, a fact of physics! Neutralise it?

SECONDARY, can we control it?

First systematic study of radiation damage in proteins:

C.C.F.Blake and D.C.Phillips 1962.
In 'Biological Effects of Ionising Radiation at the Molecular Level'.
IAEA Symposium, Vienna, P183.

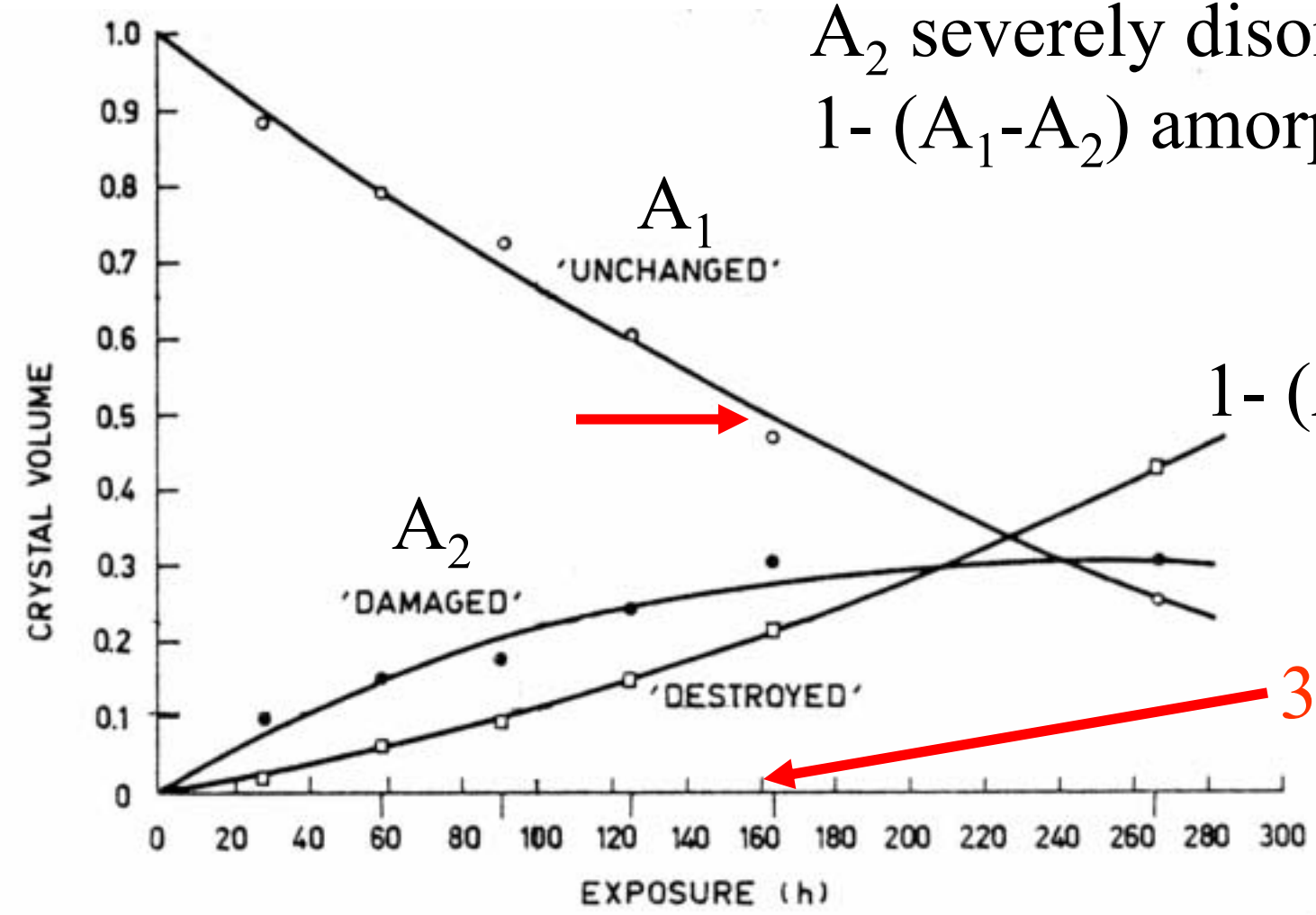
- Damage proportional to dose [Room temp].
- Each 8 keV photon absorbed disrupts ~ 70 molecules and somewhat disorders another 90.
- Damage may be structurally specific.

6 data sets of 26 hours each, 60 hour exposure, 7th

Total dose 50 Mrad (= 5 x 10^5 Gy)

Blake and Phillips model:

A_1 fraction unchanged
A_2 severely disordered
1- (A_1-A_2) amorphous



1- (A_1-A_2)

3x10^5Gy

Diffraction data after correction for damage: `There are, however, some small but significant changes in the diffracted intensitites which may indicate structural effects of the irradiation.'

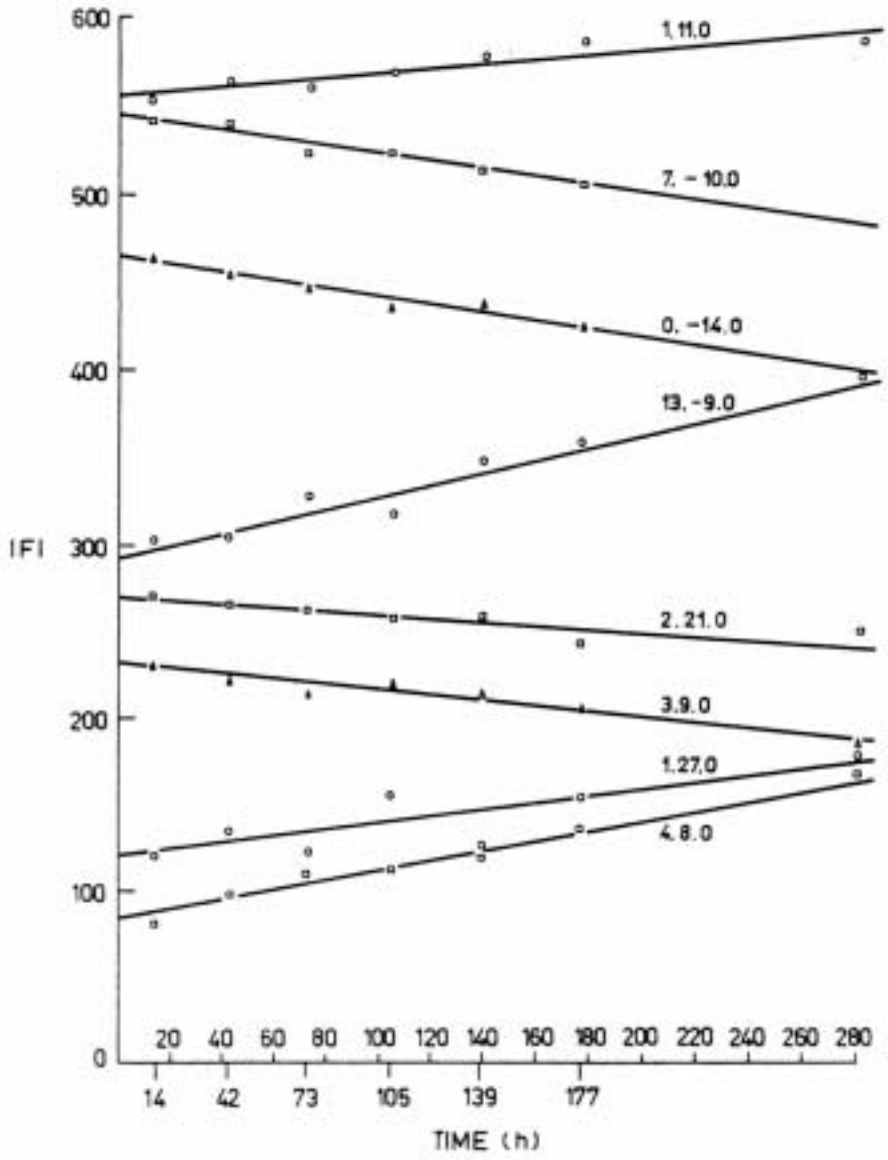
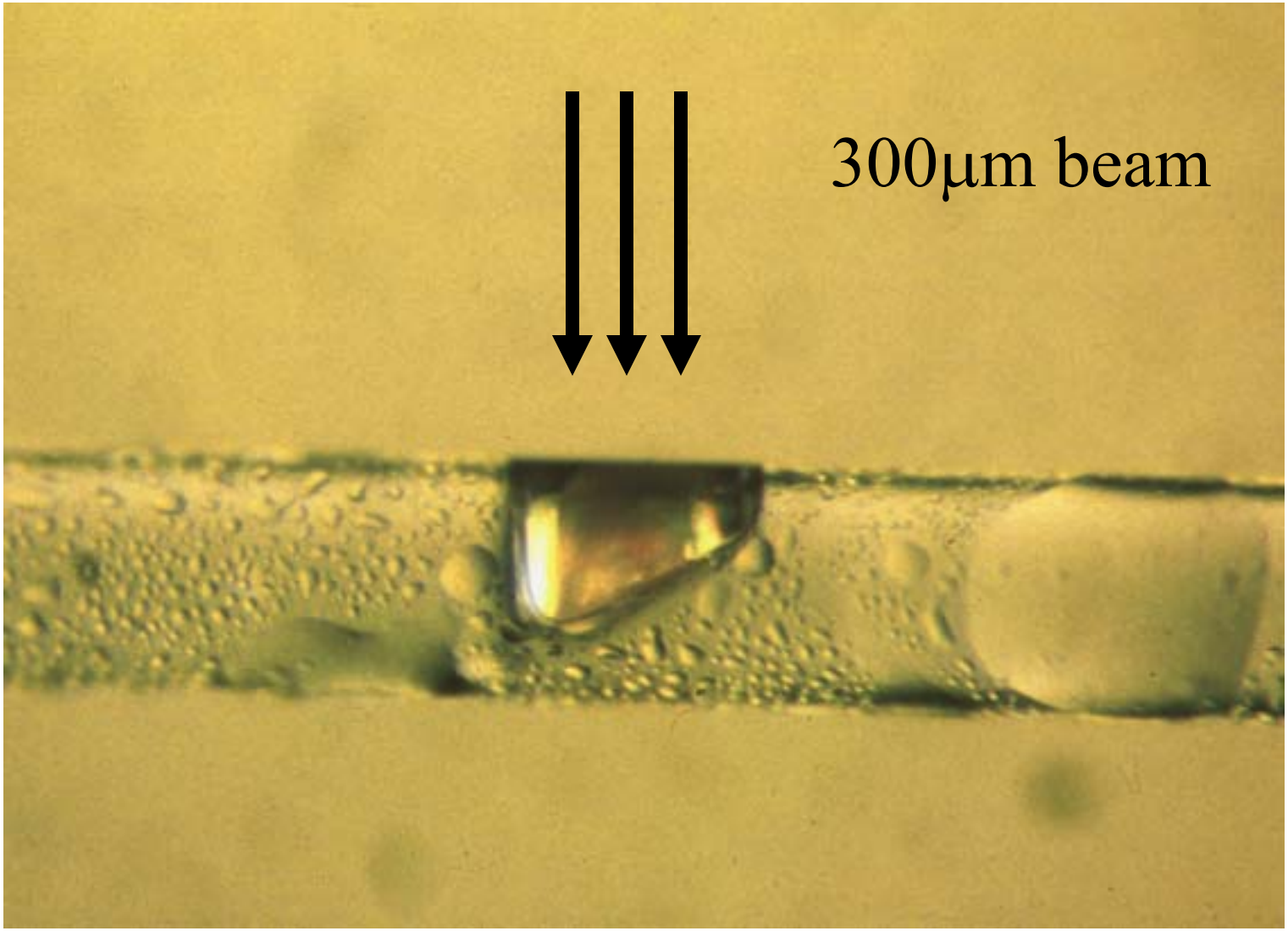


Fig.3

Behaviour of a selection of reflexions, after correction for general effects, with exposure.



Room temperature: HEWL crystal after 3 hours in a 2nd generation synchrotron beam.

Primary X-ray interaction processes with crystal and solvent.

- Thomson (Rayleigh) scattering.

  ELASTIC - no energy loss.

  Coherent – adds vectorially and gives diffraction pattern.

  Small proportion of total scattering

  BUT IT IS THE BIT WE WANT!!.

  [8% at 1Å]

Primary X-ray interaction processes with crystal and solvent.

- Compton scattering. INELASTIC.

  X-ray transfers some energy to atomic electron and a second lower energy photon is emitted. Original X-ray now has lower energy.

  Incoherent – part of X-ray background in images.

  Also a small proportion of total scattering.

  [8% at 1Å]

Primary X-ray interaction processes with crystal and solvent.

- Photoelectric effect. INELASTIC.

  X-ray transfers all its energy to an atomic electron, which is then ejected.

  Atom can then emit a characteristic X-ray or an Auger electron to return to its ground state.

  [84% at 1Å]

Note: > 90% of the beam does not interact at all

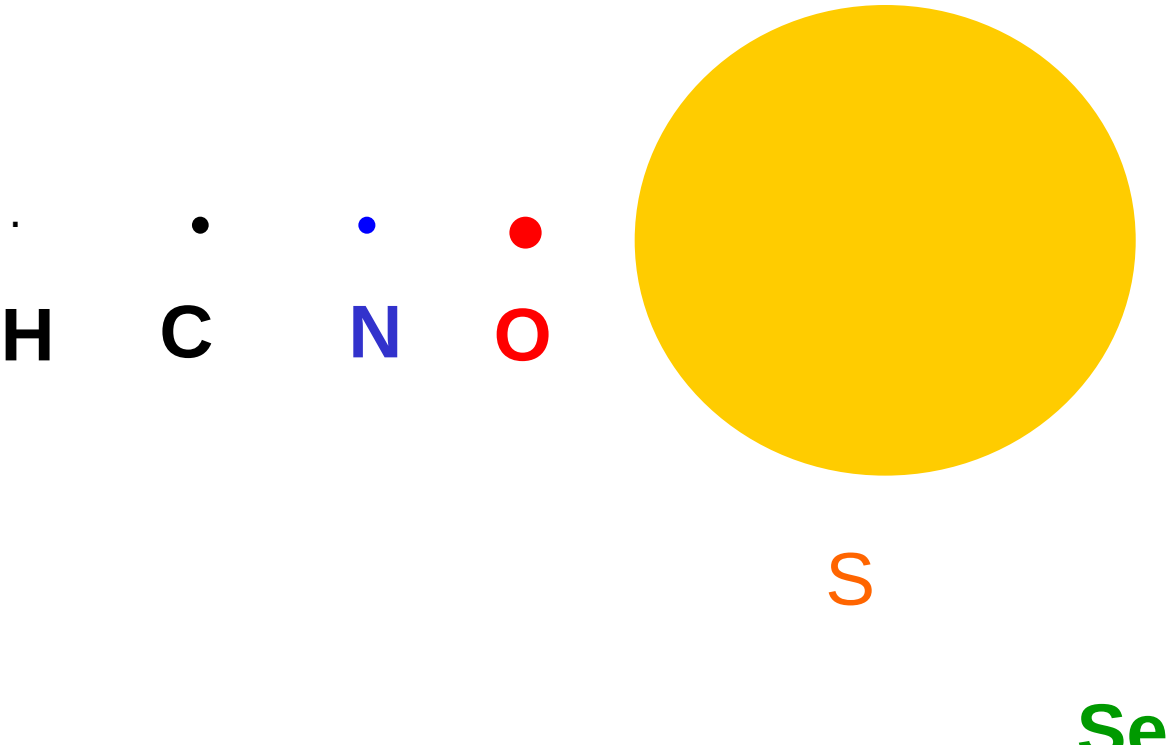
$$\sigma tot = \sigma\ pe + \sigma\ inc + \sigma\ coh$$

84% + 6% + 6%

Photoelectric Cross Section (barns /atom)

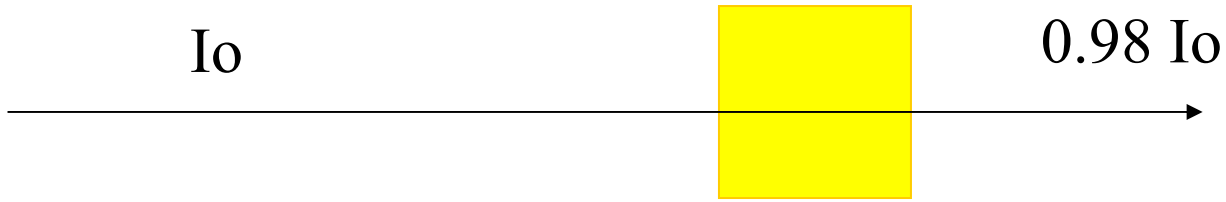
at 13.1keV

A few heavy atoms can make a big difference.

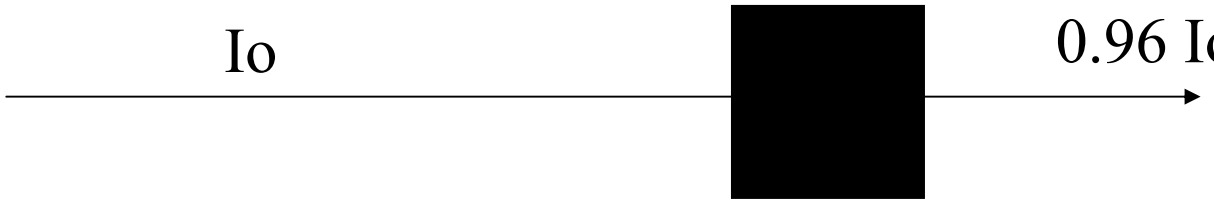


Beam absorption (λ=1Å) by a protein crystal

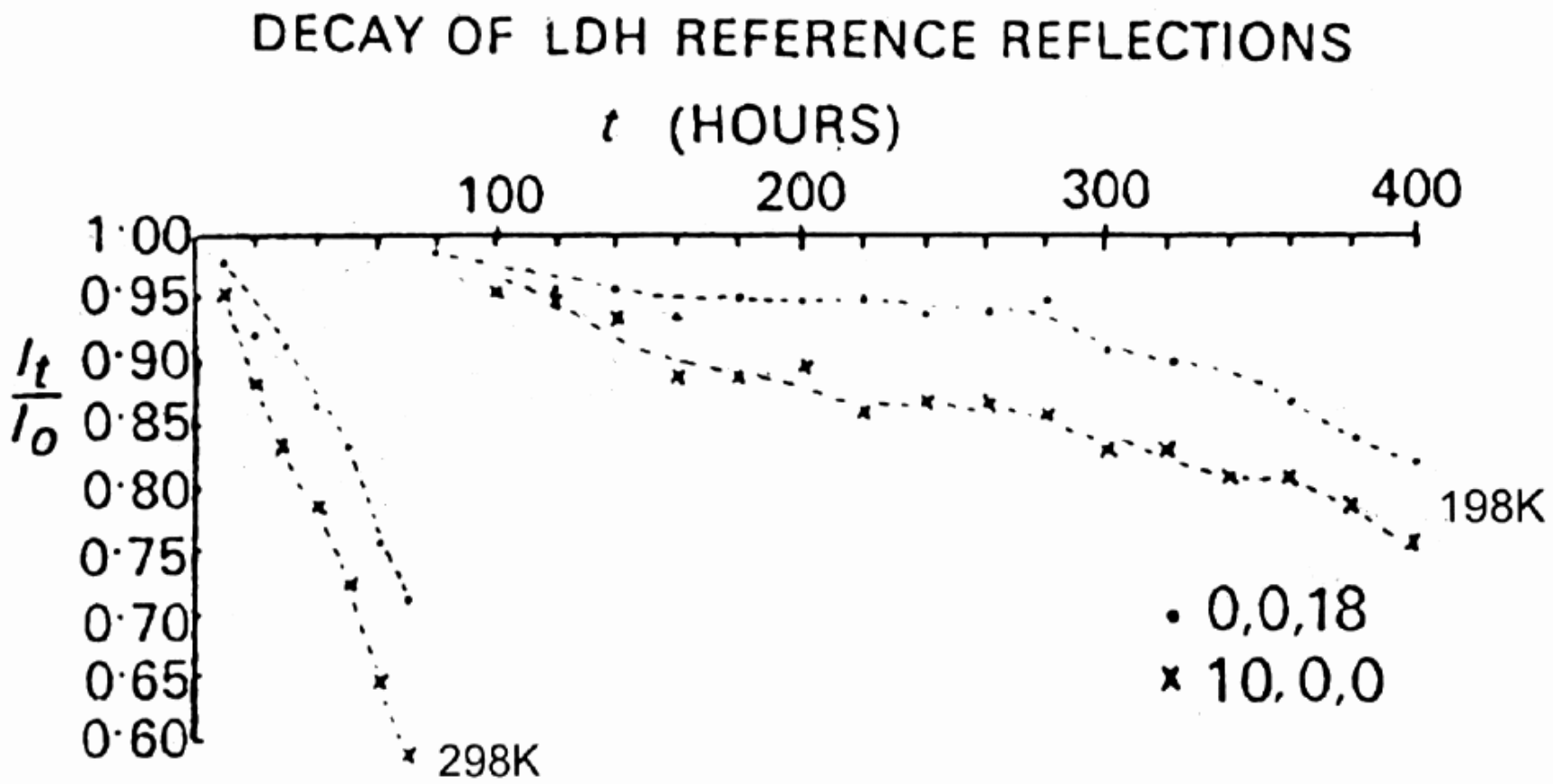
Native HEWL 100 μm thick



Mercury derivatised HEWL 100 μm thick



N.B. INCIDENT FLUX is the SAME but the absorbed dose is DOUBLE

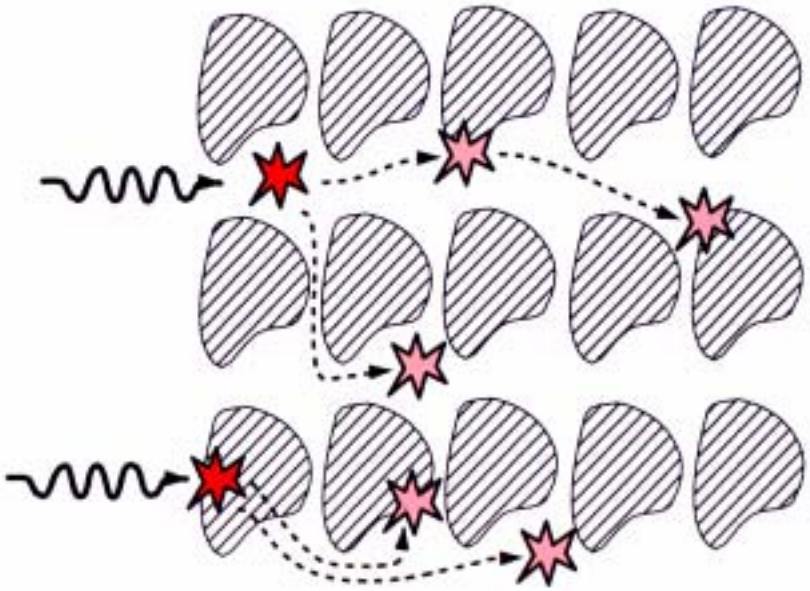


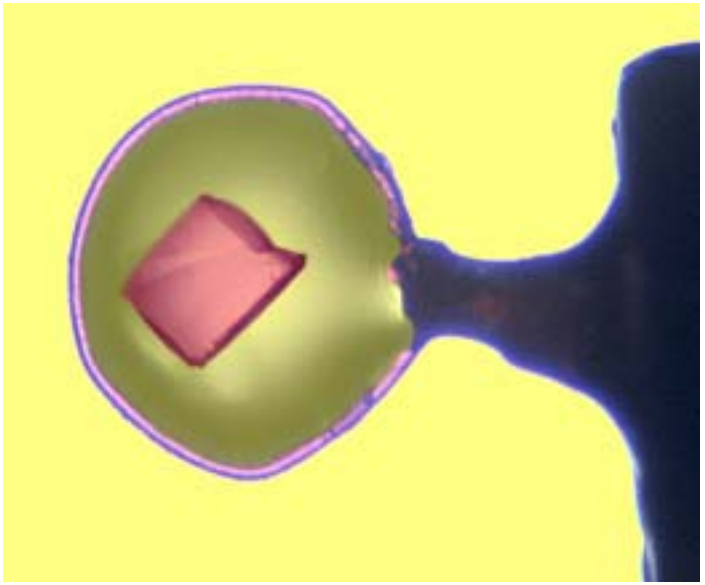
Haas and Rossmann 1970: lactate dehydrogenase

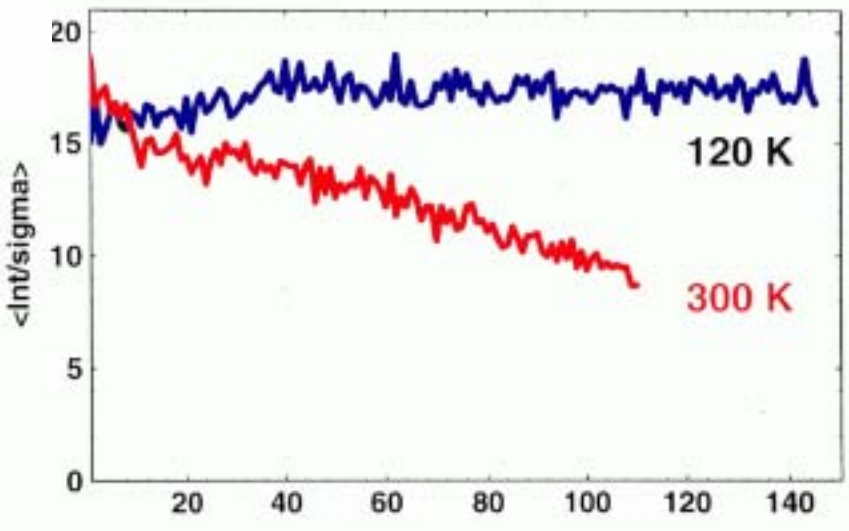
Acta Cryst B26, 998-1004.

Radiation Damage

: significantly reduced at 100K







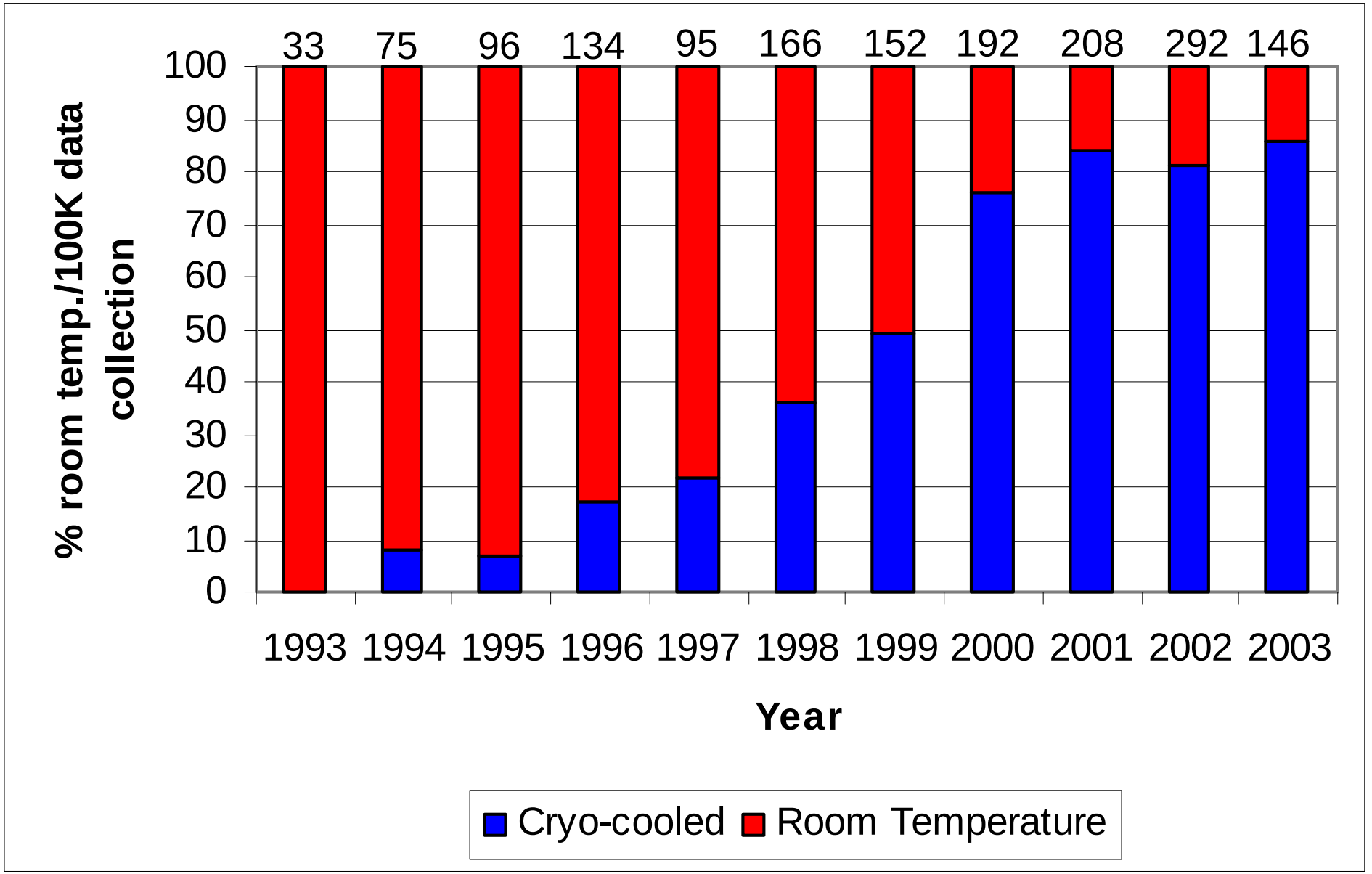
Primary:

Secondary:

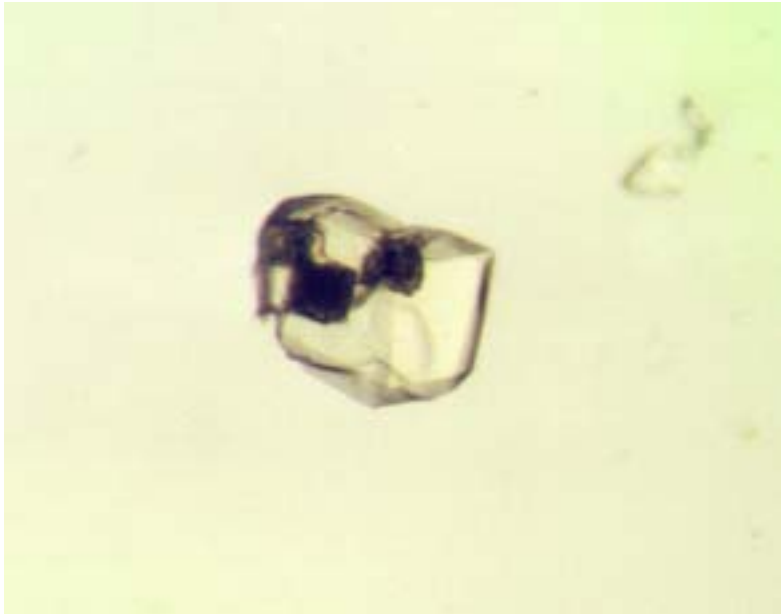
PRIMARY; inevitable, a fact of physics!

SECONDARY, can we control it?

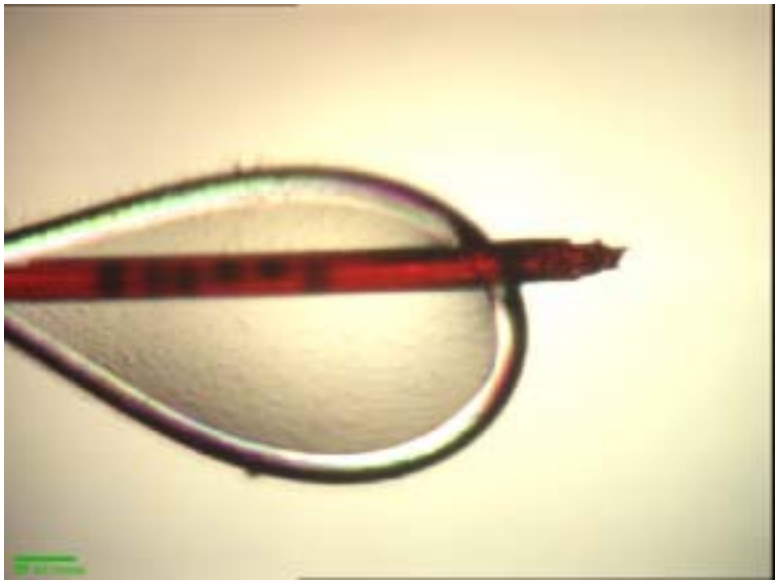
Proportions?



[Garman, Current Opinion of Structural Biology 2003, **9,** 545-551]

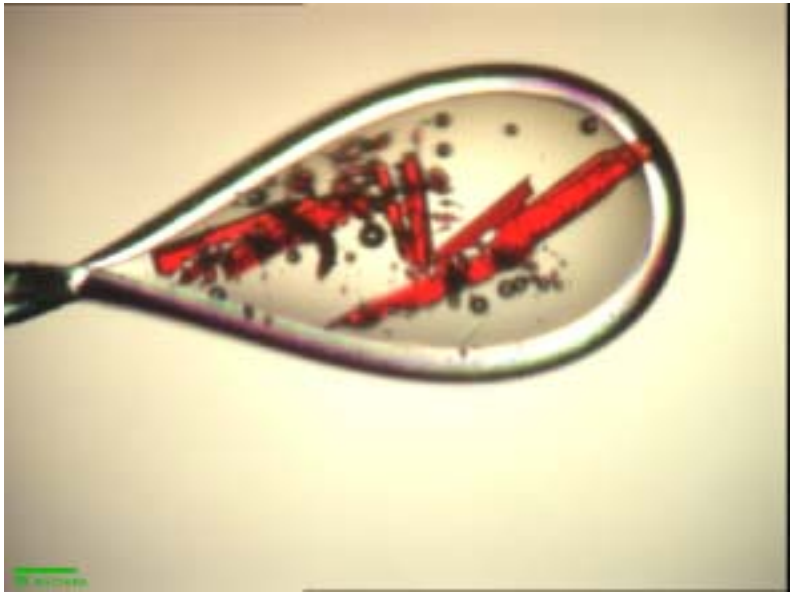


ID14-4 ESRF



Microfocus beam, ESRF

Crystals allowed to warm up after 100K data collection: crystal translated several times in beam.



[Tassos Perrakis]

The Plan:

- Radiation damage: what is it?
- **Maximum theoretical tolerable dose.**
- Why does it matter?
- Can we control it?
- Or even use it?

Maximum theoretical tolerable dose:

Theoretical radiation dose (in Gy = J kg^{-1}) limit for biological specimens at 77K.

[Henderson (1990) Proc. R. Soc. Lond. B **241**, 6-8.]

- For 100keV electrons, diffraction from protein crystals fades to half the intensity after a dose of:

5×10^7 Grays

[say **2×10^7** Grays in first part of depth-dose curve (~50μm)]

(1 Gray = 1Joule kg^{-1})

- Energy deposited by an 8keV (1.54Å) X-ray (mean penetration range 1mm): 12×10^{-16} Grays $photons^{-1}$ m^{-2}

[=**12×10^{-10}** Grays $photons^{-1}$ mm^{-2}]

- Limit achieved with a total flux of 8keV

$$\frac{2 \times 10^7}{12 \times 10^{-10}} = \mathbf{1.6 \times 10^{16}\ photons\ mm^{-2}}$$

$=10^{14}$ photons 0.1 x 0.1mm^{-2}

1995: 3rd generation synchrotron: ESRF, Grenoble.

1999: ID14-4 : **10^{12} photons s^{-1} 0.1 x 0.1mm^{-2}**

Maximum `tolerable' dose:

- `Henderson limit': **2 x 10^7 Gy.** (77K)
  - In house (with multilayers): 2.5 years, 7 x 10^7 photons/sec of 1.54 Å into 300μm^2 slits, 0.68 Gy/sec
  - SRS 9.6: 24 hours, ≈10^{10} photons/sec of 1.0 Å into 200x200μm^2 slits, 231 Gy/sec
  - ESRF ID14-4: 5 mins, 10^{12} photons/sec of 1.0 Å into 100x100μm^2 slits, 2.8 x 10^4 Gy/sec
- Limit is 70 times Blake and Phillips room temperature 1.54 Å observation of 50% I(t)/I(0) after dose of 3x 10^5 Gy

  - N.B. ABSORBED dose is what matters, NOT incident flux.
  - RADDOSE (Murray, Garman and Ravelli, *J.Appl. Cryst.* (2004) 513-522 (August issue).

Way of estimating absorbed dose, D: (Gy = J kg^{-1})

Dose rate = mass absorption coeff * photon energy * number of photons in unit time / Area

$dD/dt = (\mu/\rho)\ E\ I_{inc}$ (I_{inc}= incident flux density)

For (μ/ρ) in cm^2/g, I_{inc} in photons/s/μm^2, E in keV, t in seconds, total dose is:

$$D = (\mu/\rho)\ E\ I_{inc}\ t\ 10^{11}\ (Gy)$$

e.g. $(\mu/\rho) = 2.6\ cm^2/g$ (50% solvent), E=12keV,

A = 80 x 80 μm^2 beam cross section

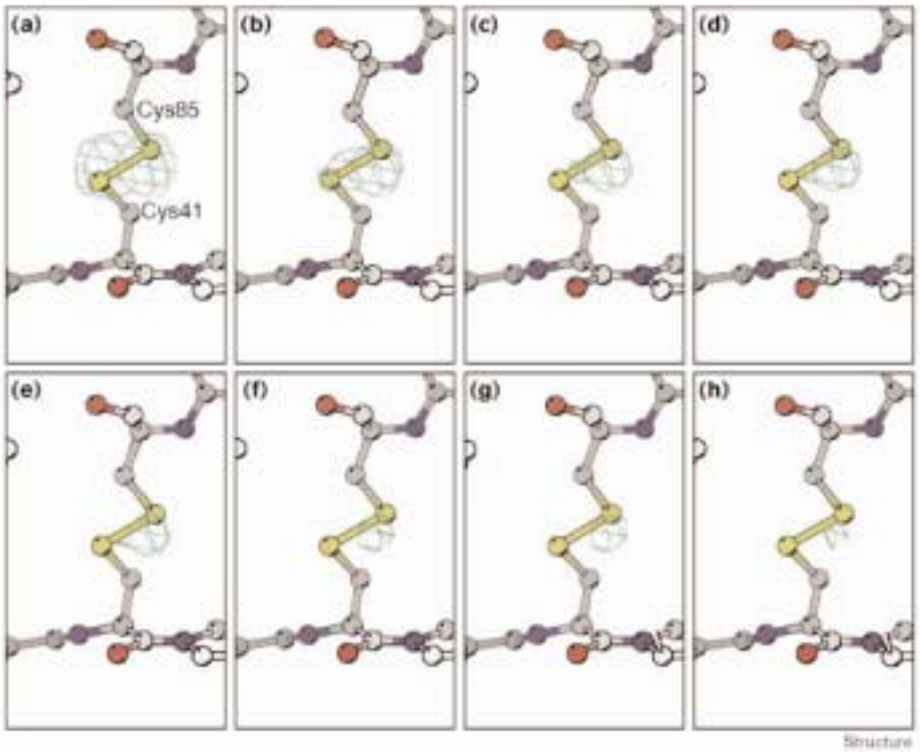
$$D = 7.8 \times 10^{-8}\ Gy/photon$$

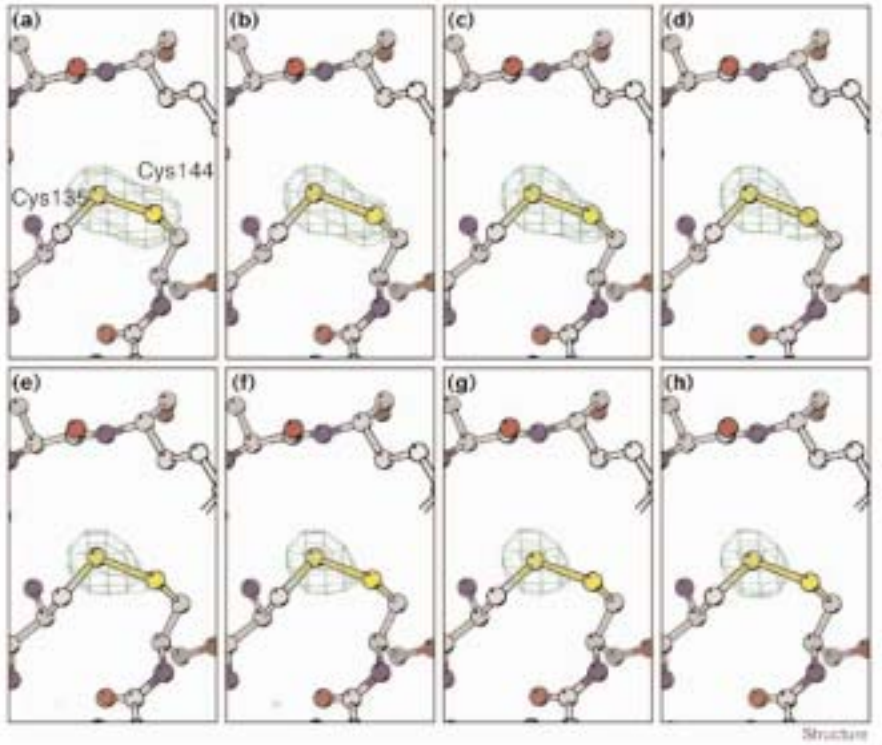
- Agrees approximately with ID14-4 observations
  - For 10^6 Gy, 1 ionisation / 20 amino acids for a 400 a.a. protein molecule. [See O'Neill, Stevens and Garman. JSR (2002) **9**, 329-332]

The Plan:

- Radiation damage: what is it?
- Maximum tolerable dose.
- **Why does it matter?**
- Can we control it?
- Or even use it?

HEWL disulphides



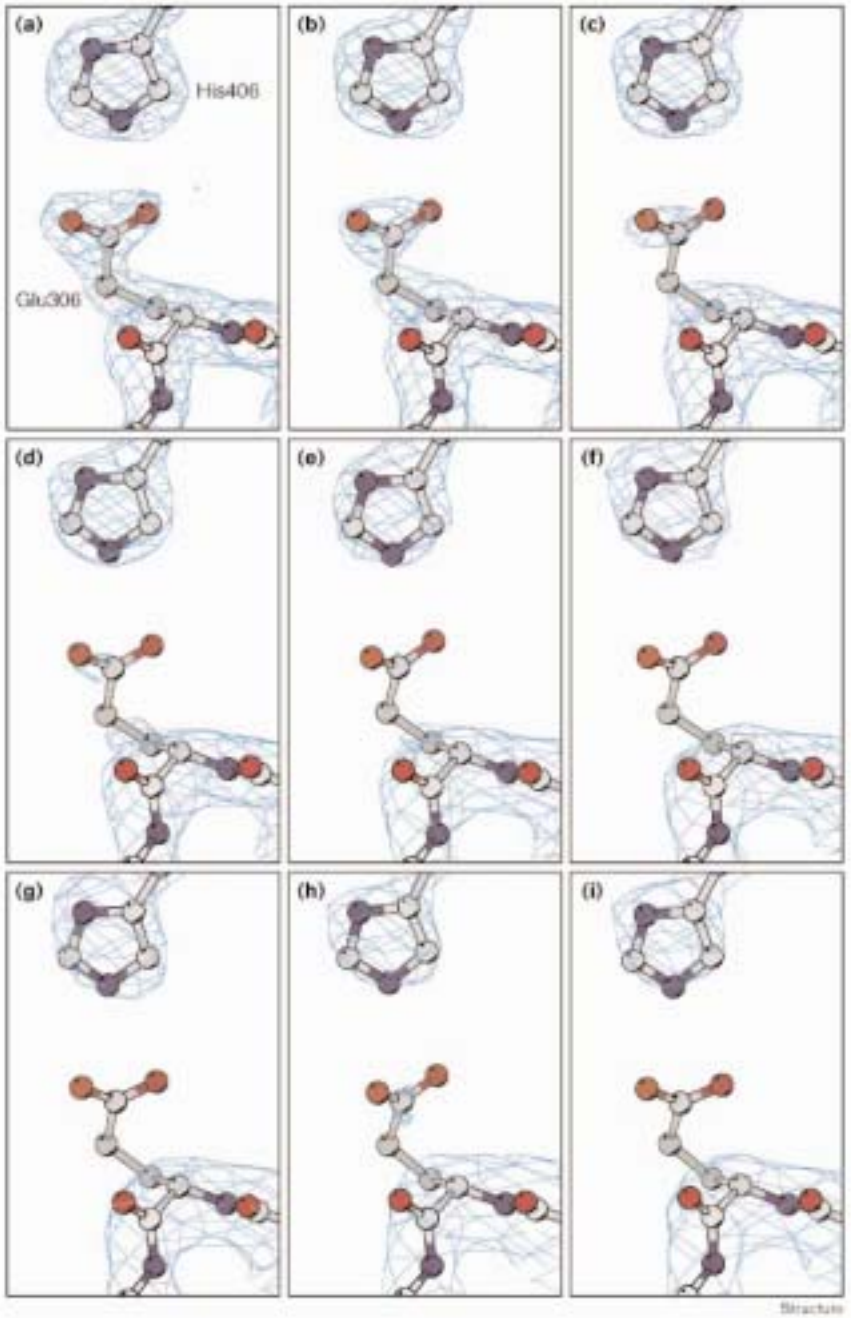


[Ravelli and McSweeney (2000)]

Acetyl cholinesterase:

TcAChE
Glu306

[Ravelli and McSweeney (2000)]



Specific structural damage observed:

- Disulphide bridges broken
- Decarboxylation of glutamate and aspartate residues
- Tyrosine residues lose their hydroxyl group
- Methionines: carbon sulphur bond cleaved

Weik *et al* (2000) PNAS 97, 623-628
Burmeister (2000), Acta Cryst D56, 328-341.
Ravelli and McSweeney, (2000) Structure 8, 315-328.

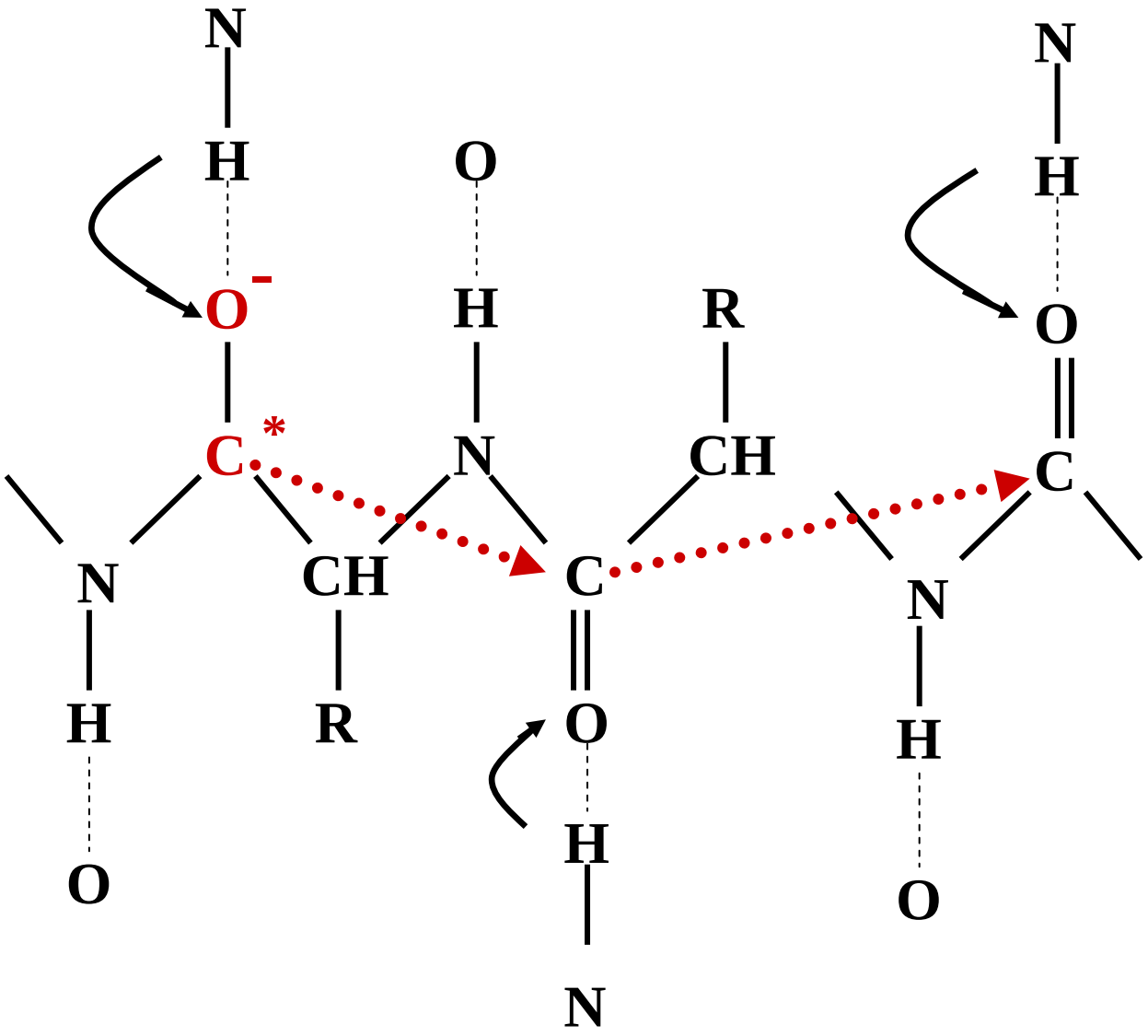
Note that if this were due to primary damage alone, damage would be in order of absorption cross sections of atoms.

DIRECT RADIATION DAMAGE. Protein Redox-

a) electron migration and trapping.

Excess electron migrates (q.m. tunnelling) along backbone – trapped at a unique C=O.

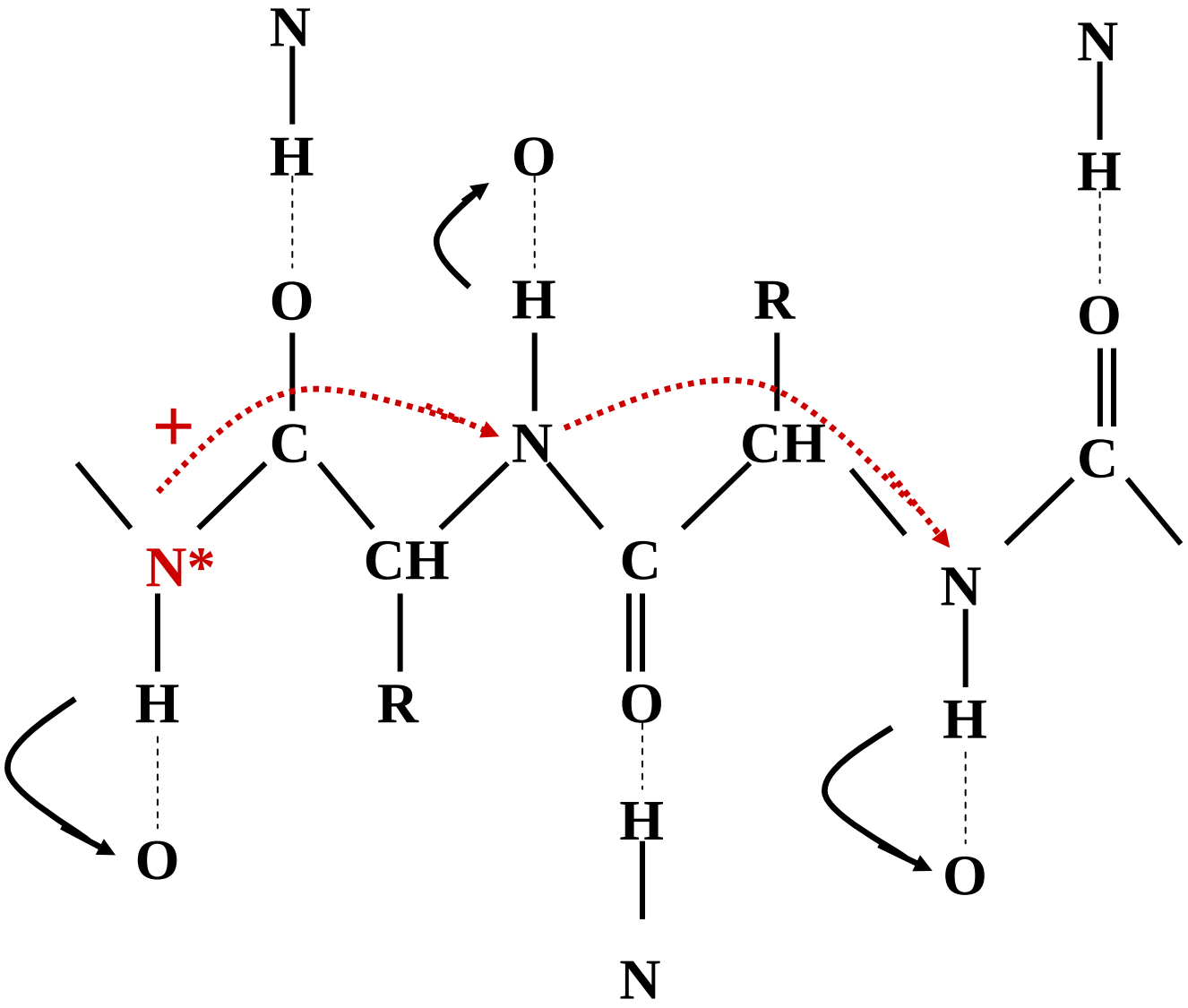
ESR: electrons mobile at 77K



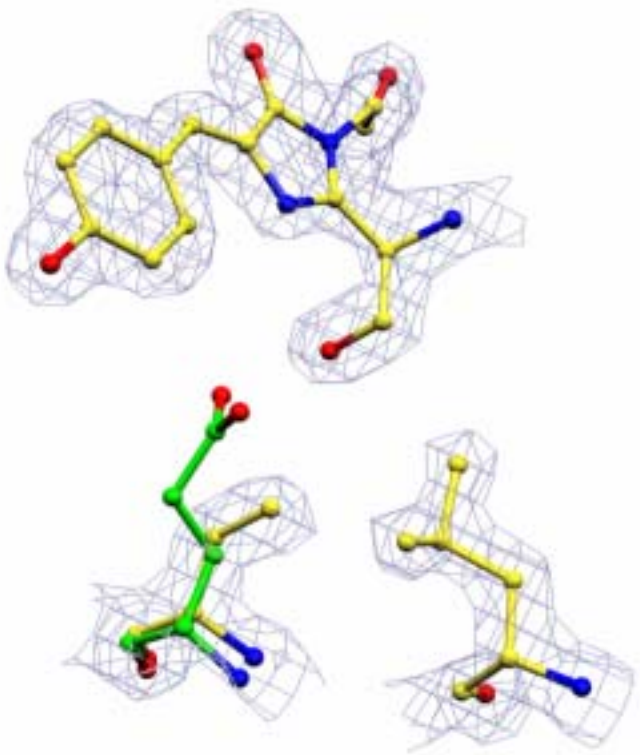
DIRECT RADIATION DAMAGE. Protein Redox-

b) proton hole migration.

Loss of proton from the cationic site.



Radiation damage affects biological results



- GFP: Decarboxylation of Glu 222 is part of the protein mechanism, but is indistinguishable from radiation damage at the synchrotron.

[van Thor, Gensch, Hellingwerf and Johnson, *Nat. Struct. Biol.*, (2002) **9(1)**]
Also bacteriorhodopsin, Matsui *et al.*, *JMB* (2002) **324**, 469-481.

Manifestations of Radiation Damage

- Loss of diffraction: incomplete data from crystals
- Specific Structural damage
- WRONG BIOLOGICAL INFORMATION
- `Pollutes' good ultra-high resolution data
- **Failure of structure determination (MAD) due to creeping non-isomorphism – anisotropic cell expansion and structural changes DURING experiment.**

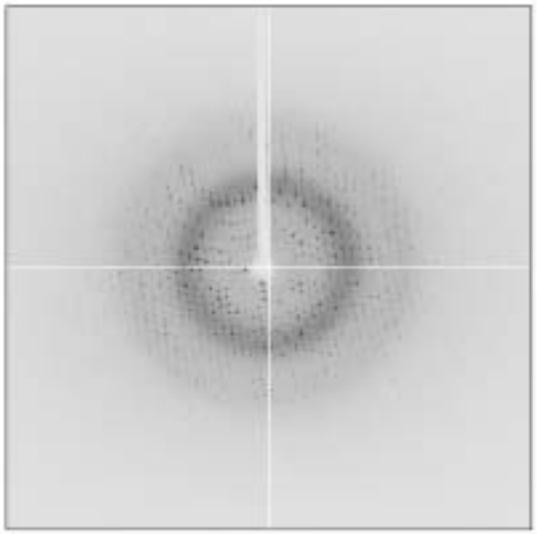
The Plan:

- Radiation damage: what is it?
- Maximum tolerable dose.
- Why does it matter?
- **Can we control it?**
- Or even use it?

PROBLEM: how do we know that we are making any difference?

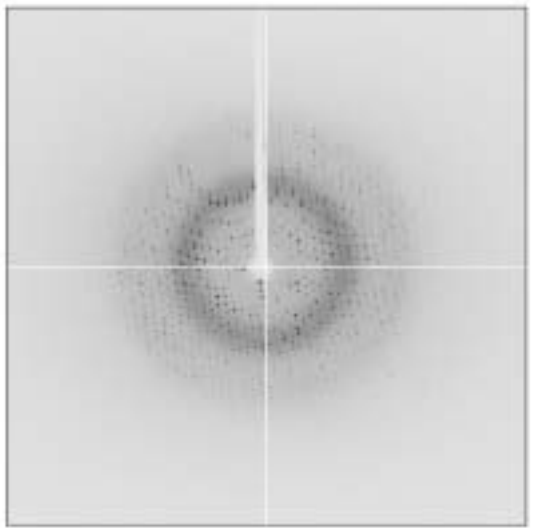
- In order to investigate the effects of various parameters on the radiation damage process, we need a radiation damage METRIC which is preferably ON-LINE during the diffraction experiment.

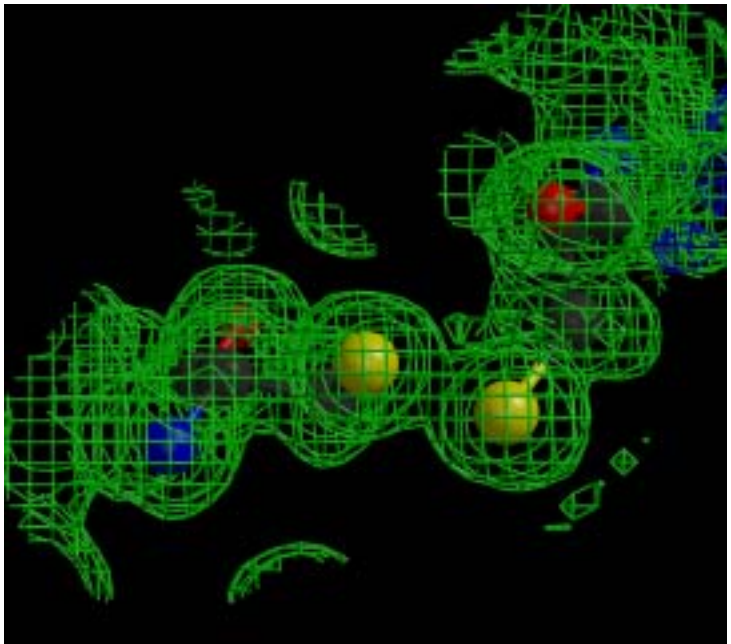
PROBLEM:
Specific damage occurs before diffraction pattern is compromised.

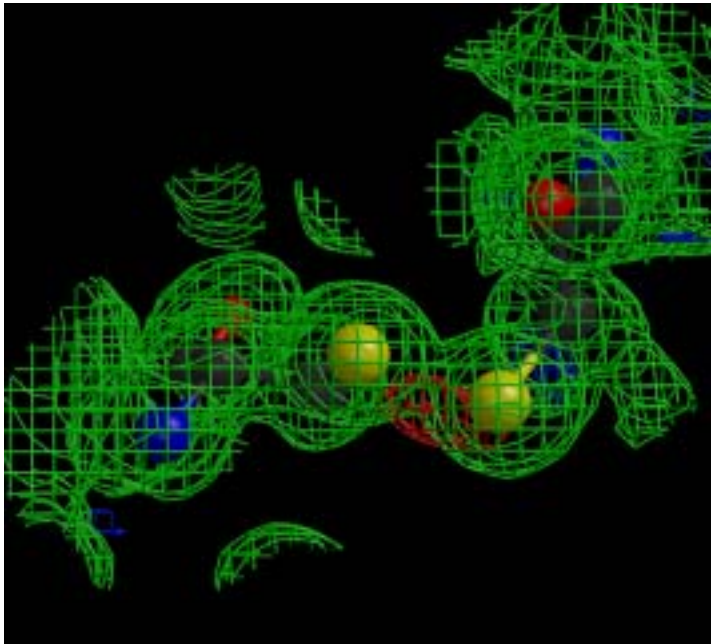


before

after







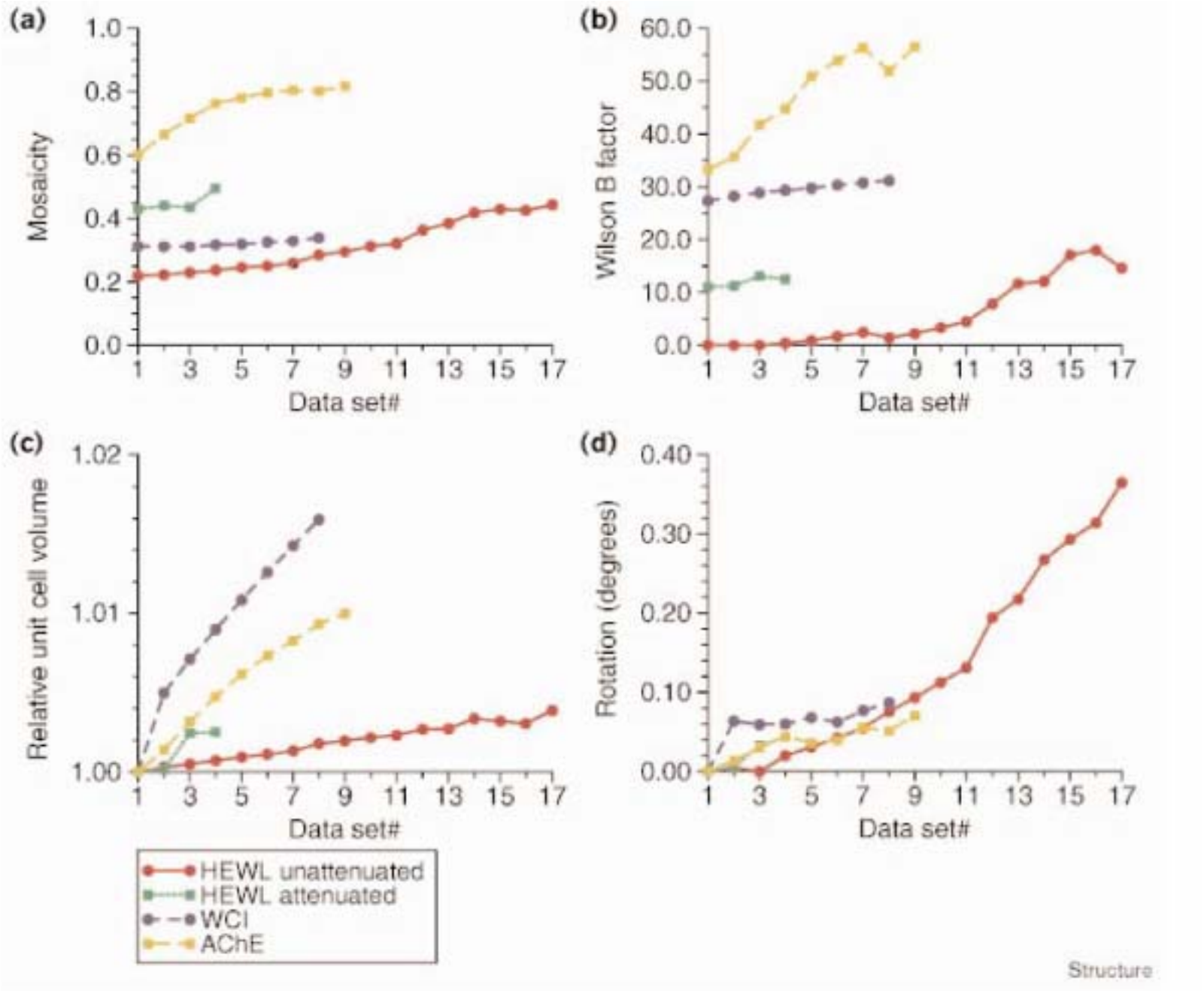
How to Quantify Radiation Damage?

- B factors
- Mosaicity
- R_{merge}
- $I / \sigma(I)$ or resolution limit
- Specific damage in electron density maps:

  broken disulfides, decarboxylation (Asp and Glu), loss of hydroxyls (Tyr).
- Unit Cell expansion a) function of dose

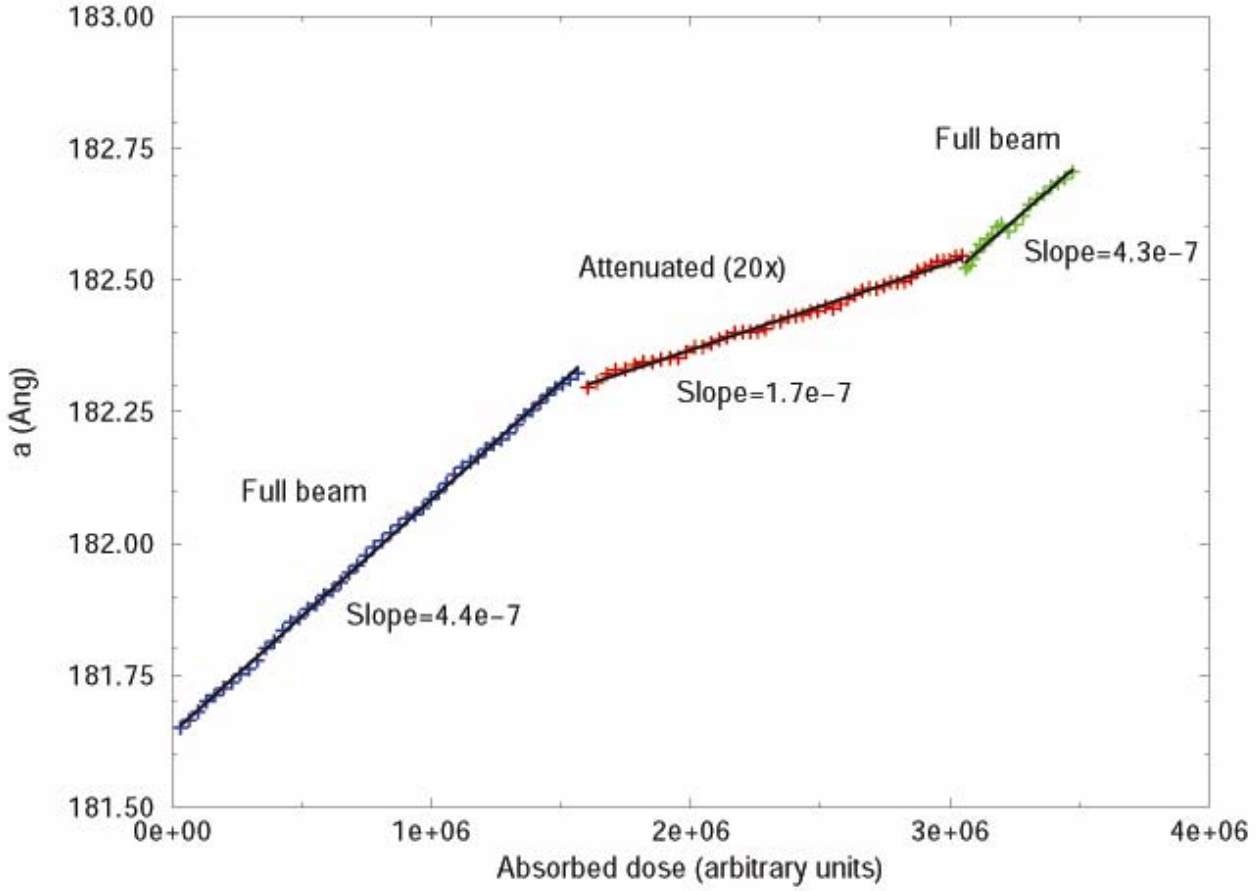
  b) function of cryogen temperature

Could this be an on-line damage metric? [Ravelli and McSweeney, 2000]

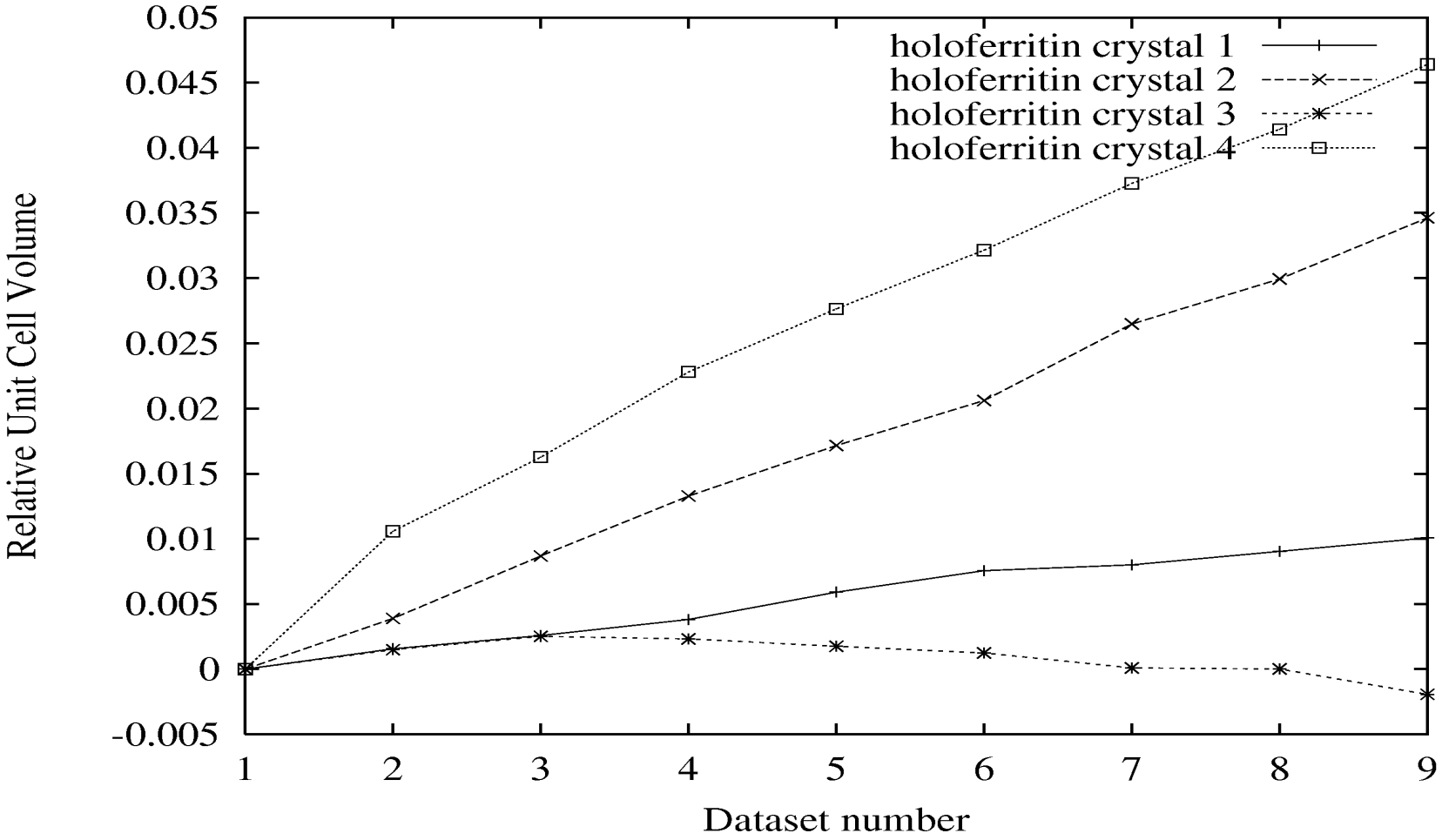


Ravelli and McSweeney, Structure (2000) 8, 315

Cell Expansion – Ferritin



Raimond Ravelli *et al* – JSR (2002) **9**, 355-360. **Note DOSE RATE effect.**



Holoferritin

F432, a=183Å, max 4.5% expansion

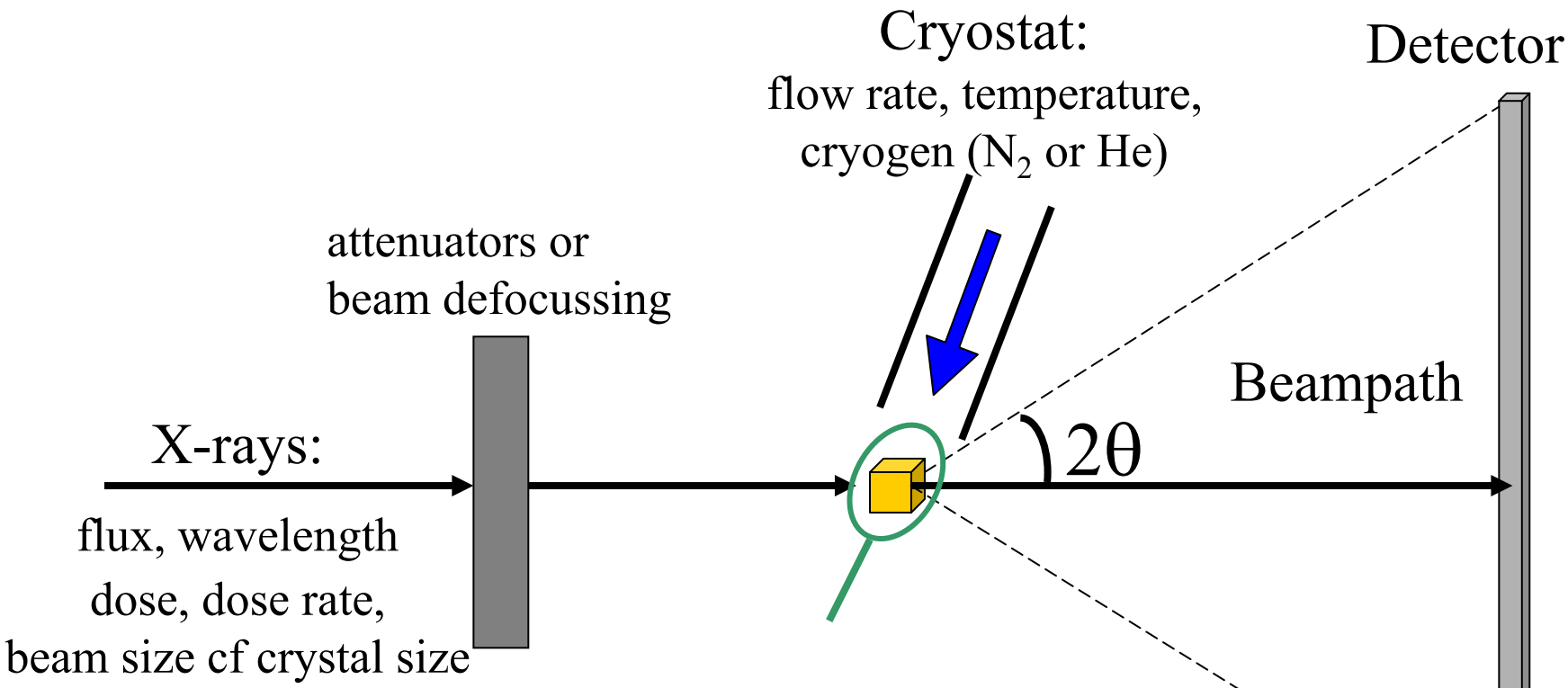
What can we do to mitigate it? Relevant parameters include:

A) External variables:

- Incident beam conditions: wavelength, dose, dose rate, flux.
- Cooling regime: Nitrogen or Helium, temperature, flow rate.

B) Physical and chemical environment of crystal in loop:

- Cryoprotectant agent choice and concentration, solvent content of crystal, S/V of crystal.



Crystal in loop:

heavy atom content (Se, S etc), solvent content,
solvent composition, crystal size and S/V,
amount of residual liquid around crystal,
choice and concentration of cryoprotectant agent,
time in cryobuffer, flash-cooling method (stream or liquid),
cryogen used to flash-cool, amount of crystal manipulation,
humidity, speed of experimenter when flash-cooling.

[Garman, Current Opinion of Structural Biology 2003, **9,** 545-551]

Work so far / ongoing:

- Lower the cryogen temperature? 40K? 16K? 140K
  - Garman Acta D (1999)**D55**, 328.
  - Hanson *et al*, JAC (1999)**32**,814 and JSR (Nov 2002),
  - Teng and Moffat JSR (2000)**7**,315 and (2002)**9**,198.
  - **Phase transitions:**
  - Weik *et al*, Acta D (2001)**D57**, 566.
- Lower the wavelength? Lots of anecdote.
  - Arndt, JAC (1984)**17**, 118.
  - Gonzales and Nave (1994) Acta Cryst **D50**, 874.
- Change/ regulate the dose/dose rate regime?
  - Teng and Moffat JSR (2000)**7**,315-328
  - Sliz and Rosenbaum, Structure (2003) **11**, 13-19. Damage depends only on absorbed dose and there is no evidence for any dose rate effect up to 10^{15}p/s/mm^2
  - Ravelli et al, JSR, (2002) **9**, 355-360.

- Effect on MAD/SAD
  - Rice *at al* Acta Cryst (2000) **D56**, 1413.
- Minimum crystal size
  - Gonzales and Nave (1994) Acta Cryst **D50**, 874.
  - Glaeser *et al* Biophys J., (2000)**78**, 3178
  - Sliz and Rosenbaum, Structure (2003) **11**, 13-19
- Beam heating.
  - Kuzay *et al*, Acta Cryst (2001)**D57**, 69
  - Nicholson *et al*. NIMPR (2001), **A467-468**, 1380
  - Weckert *et al*. JSR (2002) **9**, 368-374.
  - Snell *et al*, JSR (2002) **9**, 361-365.
  - Kriminski *et al*, Acta Cryst (2003)**D59**, 697-708
- Remove oxygen? Nothing yet.
- **8 more papers coming out in JSR Nov 2004 from RD3.**
- **Add radical scavengers?**

Need for **systematic statistically significant** experiments.

Scavengers at cryotemperatures: Rationale

- Crystals are usually cooled to 100K to reduce the mobility of free radicals.
- The existence of specific damage at this temperature shows that some species are still mobile.
- Therefore free-radical scavengers may be able to react with these species and reduce their mobility and reactivity, protecting the crystal from specific damage.

Scavengers: make radicals less reactive, remove `wandering' electrons.

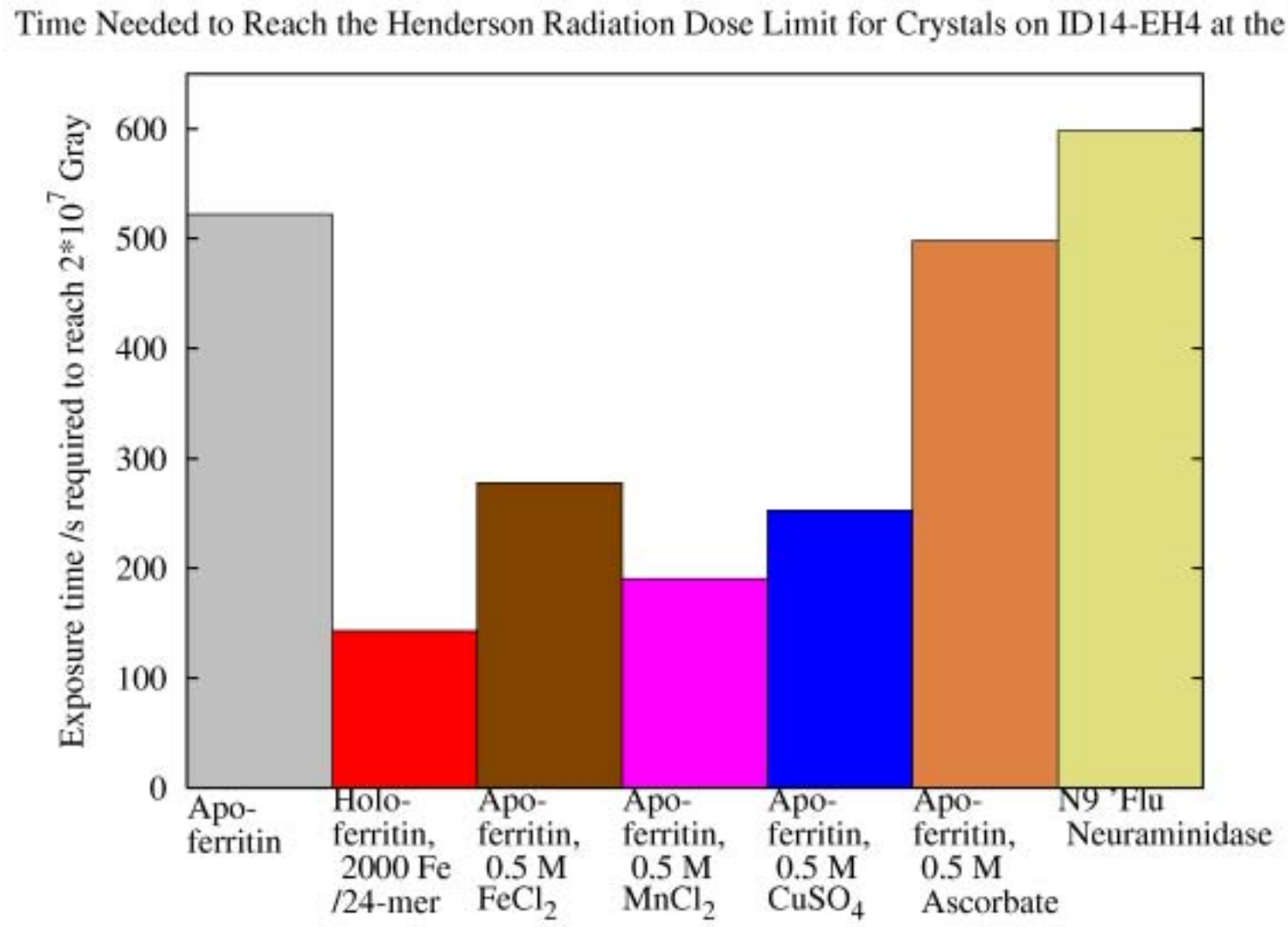
- Styrene used in 1974 for Immunoglobulin crystals. [Zaloga and Sarma, Nature, **251** pp551-552, 1974]

- Ascorbic acid
- Cysteine
- Glutathione
- HEPES, Tris
- Ethanol

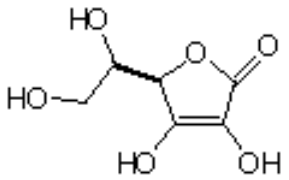
- $CuSO_4$,
- $FeCl_3$,
- $MnCl_2$
- glucose
- Ethylene glycol

• Spin Traps – DPMO and TEMP, used in ESR to capture short-lived species

Effect of metal-containing scavengers on crystal absorption

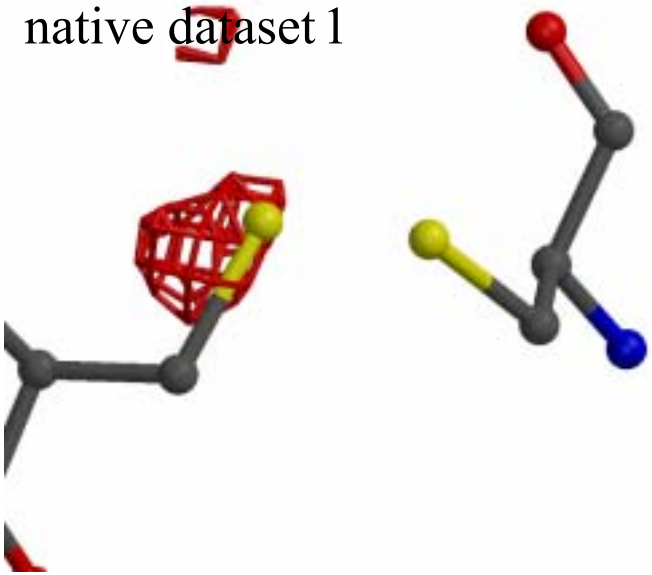


Ascorbate as a Scavenger

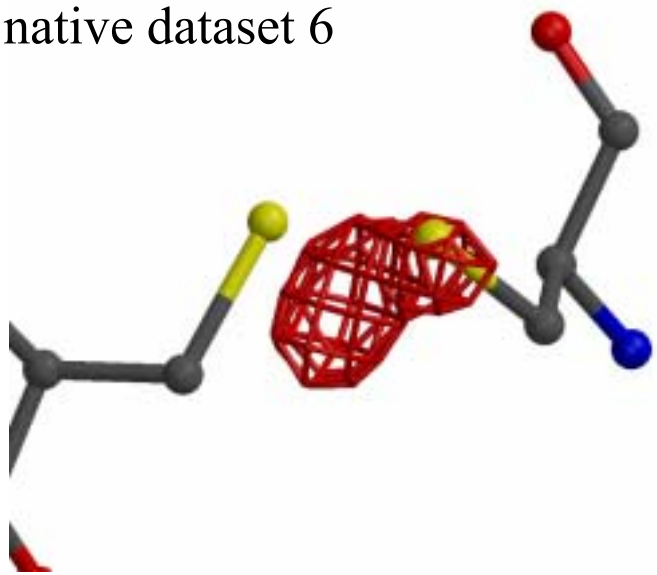


- Sodium ascorbate. Cocrystallized with HEWL at 0.5 M in NaCl pH 4.6
- Successive data sets taken from co-crystallized and “native” crystals at ID14-EH4 at the ESRF.
- Comparison of maps
- Microspectrophotometer data

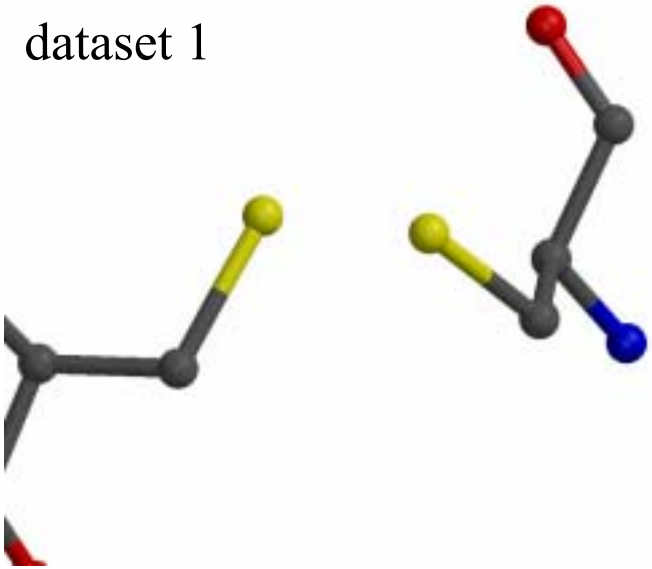
(a) HEWL
native dataset 1



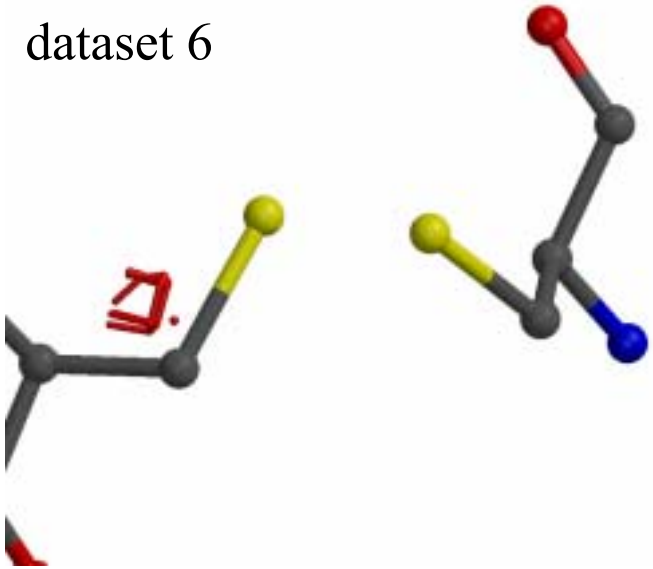
(b) HEWL
native dataset 6



(c) HEWL
ascorbate
dataset 1



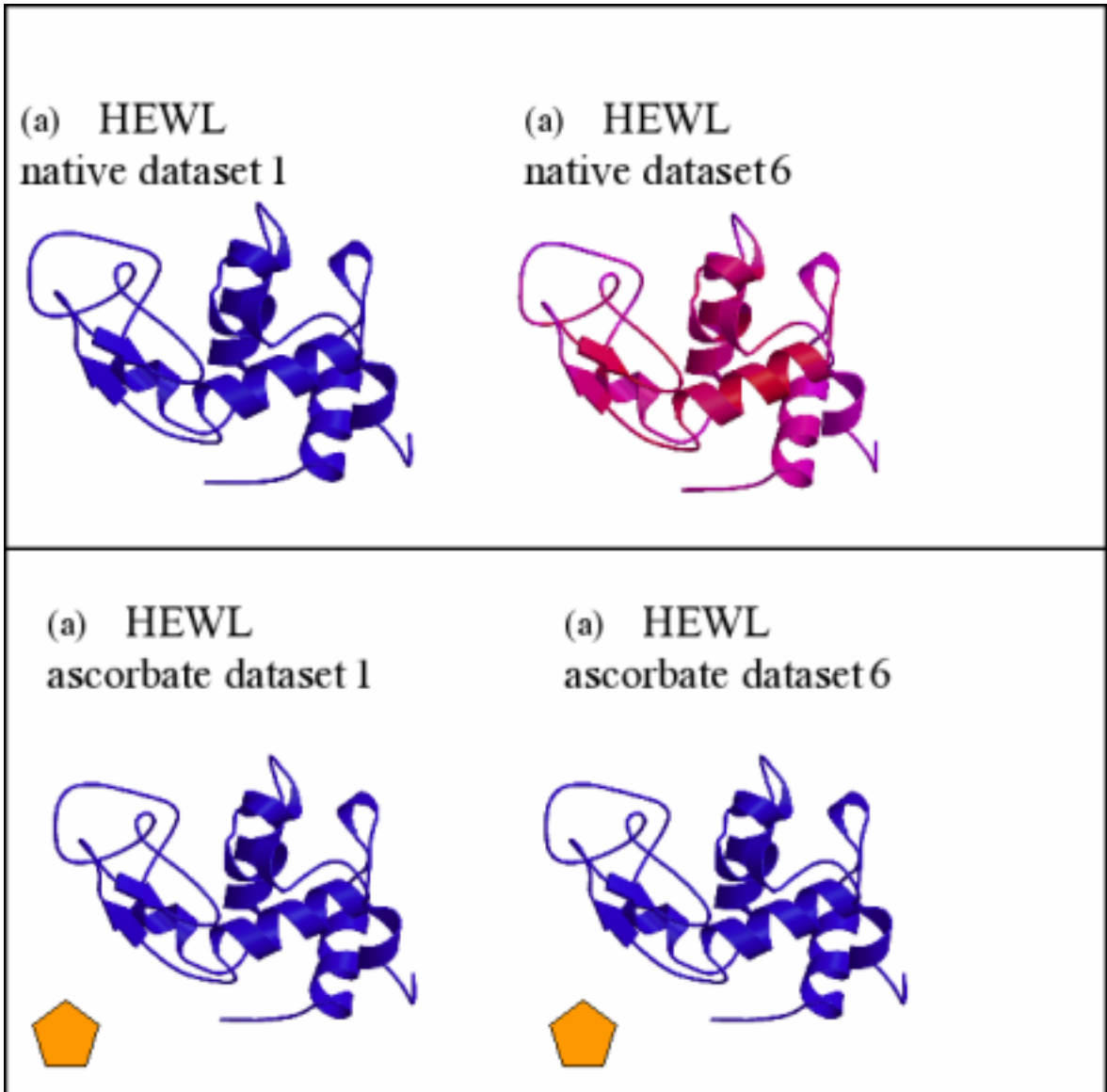
(d) HEWL
ascorbate
dataset 6



Change in atomic B factors of refined structures with radiation load.

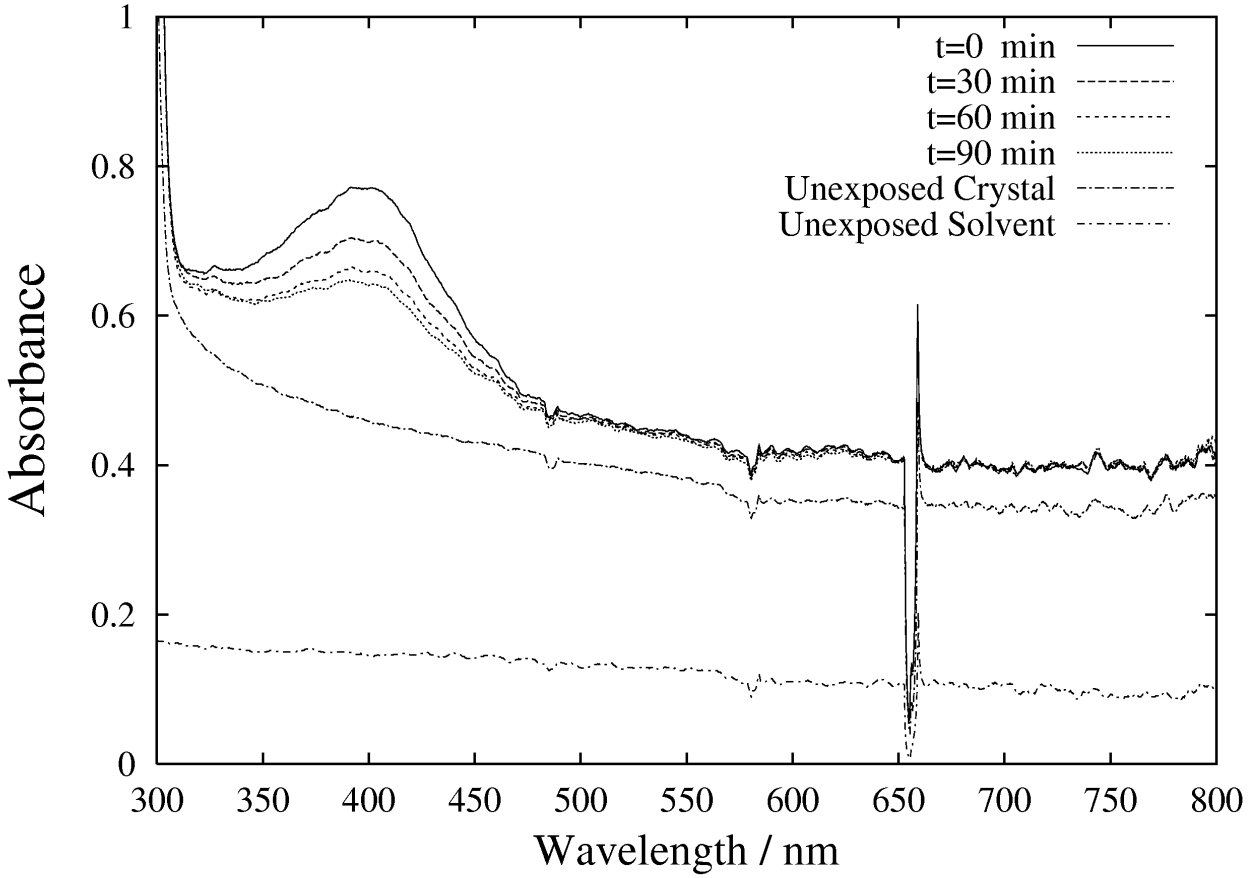
No increase in temperature factor

70 % increase in temperature factor

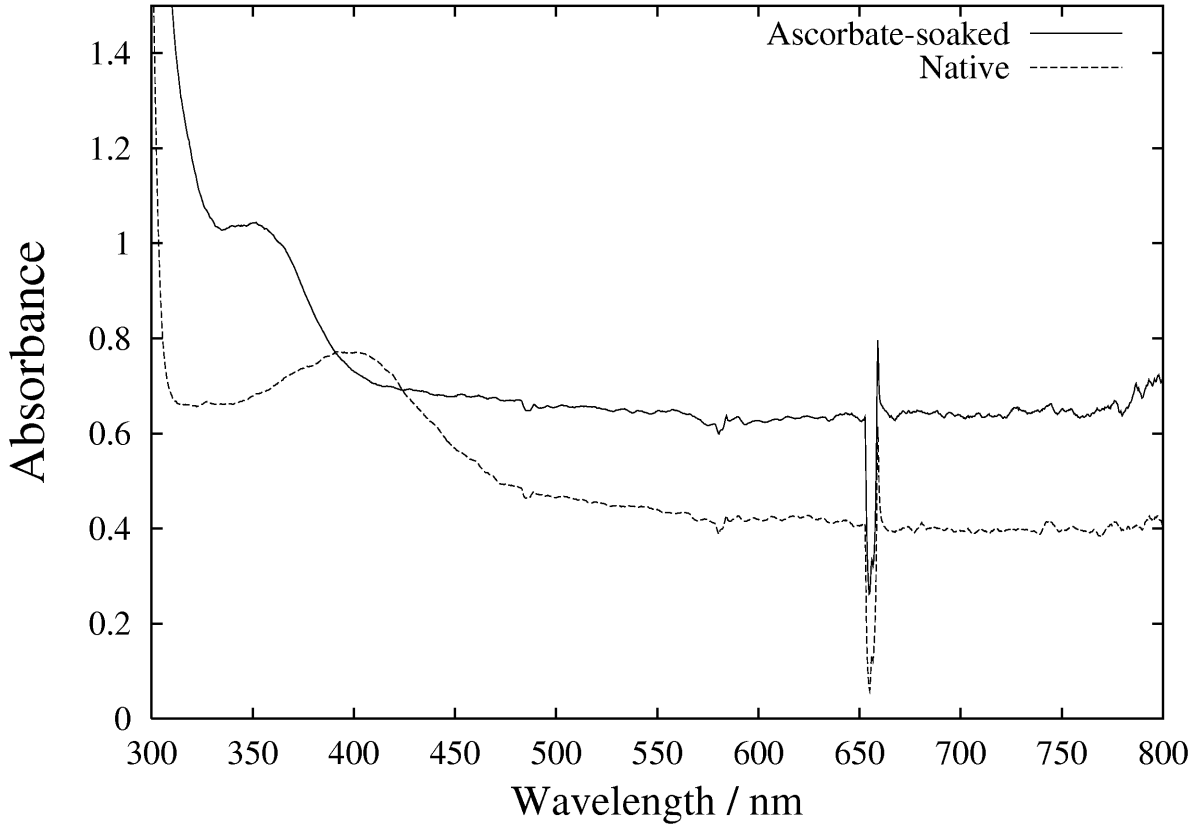


Microspectrophotometer

All measurements at 100K. HEWL crystal, exposed for 300s on ID14-EH4. Relaxation of disulfide radical anion R-SS-R^{-} ?



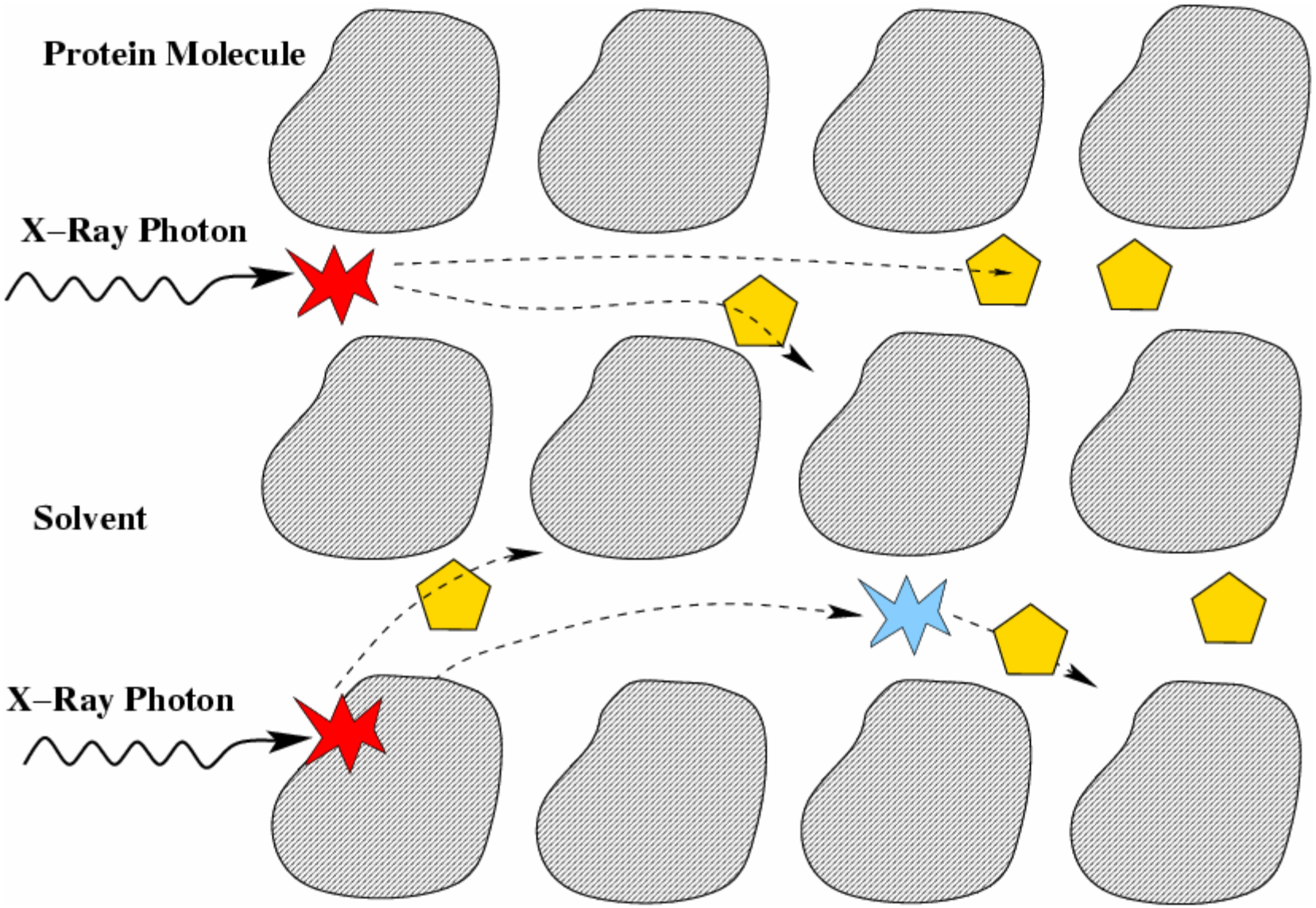
HEWL crystal on
Microspectrophotometer after 300s
Unanattenuated beam at ID14-EH4



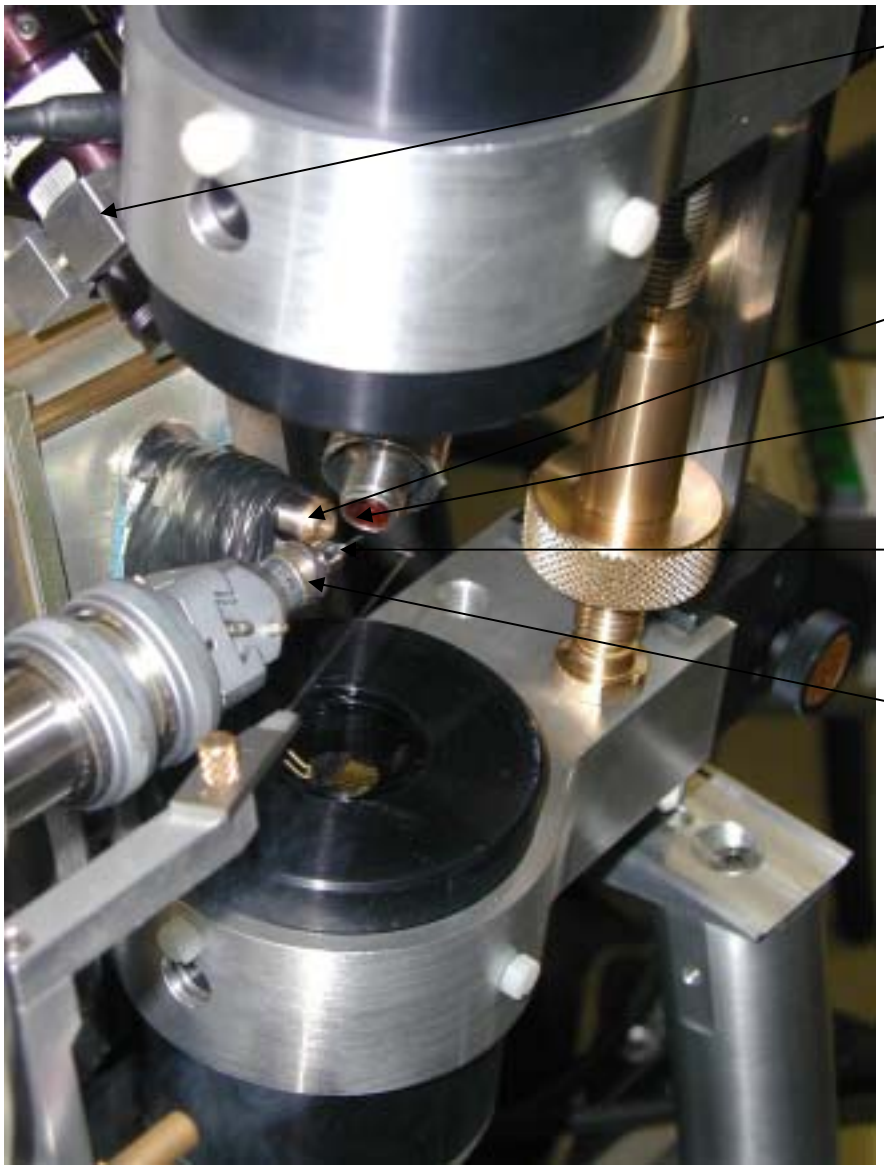
Ascorbate

- How does this work?
- Ascorbate is an OH radical scavenger and an anti-oxidant which can restore oxidised radicals by donating an electron.
- What is the mechanism?
- $RS^{\bullet} + Ascorbate^{-} \Rightarrow RS^{-} + H+$ ascorbyl radical
- radical + ascorbate $\Rightarrow$ ascorbyl radical
- More experiments with different scavengers and other protein crystals are required.
- This is of potential interest to ALL protein crystallographers

(Murray and Garman, JSR, 2002, 9,347)


Protein Molecule
X–Ray Photon
Solvent
X–Ray Photon

On-line microspectrophotometer. ESRF, ID14.4



Alignment!

The Plan:

- Radiation damage: what is it?
- Maximum theoretical tolerable dose.
- Why does it matter?
- Can we control it?
- **Or even use it?**

For phasing and new experiments……

- RIP: Ravelli *et al*, Structure (2003),

- Enzyme mechanism pathways

[e.g. HRP, Nature (2002) 417, 463-468.

Bacteriorhodopsin, Matsui et al., JMB (2002) 324, 469-481.]

Structural determination of reactive intermediates in the horseradish reaction cycle.

Gunnar Berglund, Uppsala, Sweden

Utilising radiation damage for enzyme mechanism studies.

HRP intermediates: originally generated in situ and trapped cryogenically. X-rays cause reduction of the oxidised catalytic centre. Novel irradiation regime used.

Found that catalysis could be driven by X-rays in redox enzymes.

Berglund GL, Carlsson GH, Smith AT, Szobe, H, Hendriksen A, Hadju J. Nature (2002) 417, 463-468.

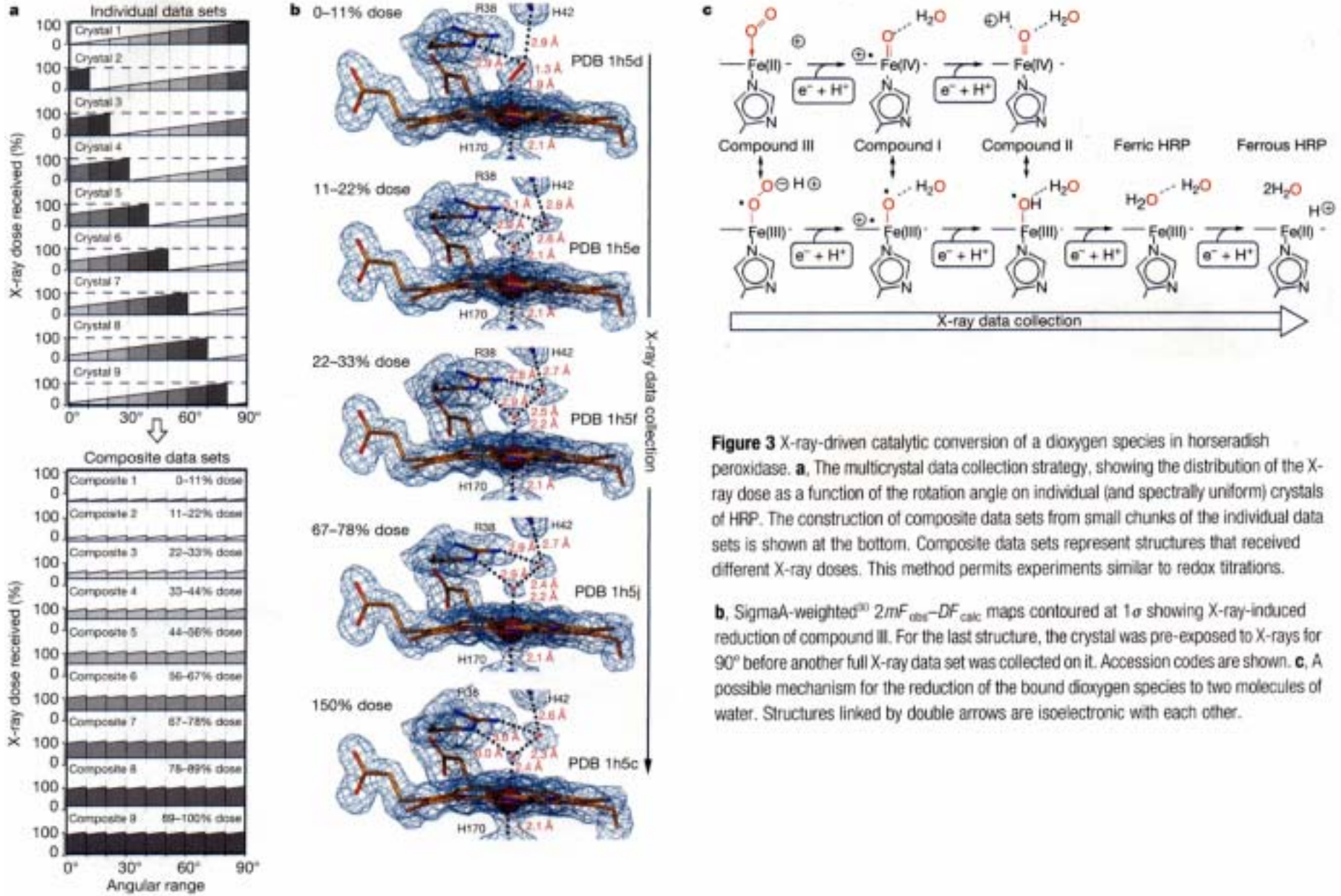


Figure 3 X-ray-driven catalytic conversion of a dioxygen species in horseradish peroxidase. **a**, The multicrystal data collection strategy, showing the distribution of the X-ray dose as a function of the rotation angle on individual (and spectrally uniform) crystals of HRP. The construction of composite data sets from small chunks of the individual data sets is shown at the bottom. Composite data sets represent structures that received different X-ray doses. This method permits experiments similar to redox titrations.

b, SigmaA-weighted[30] $2mF_{obs}-DF_{calc}$ maps contoured at 1σ showing X-ray-induced reduction of compound III. For the last structure, the crystal was pre-exposed to X-rays for 90° before another full X-ray data set was collected on it. Accession codes are shown. **c**, A possible mechanism for the reduction of the bound dioxygen species to two molecules of water. Structures linked by double arrows are isoelectronic with each other.

Current status: radiation damage in protein crystals

- Understand a lot more than five years ago, but still not nearly enough…
- Understand how to do experiments better.
- Unit cell is not a good general on-line metric.
- Scavengers: preliminary results show they may help but not the factors of 10 we need.
- Research has prompted some exciting new approaches.
- Experiments now reflect a more `non-anecdotal' approach, but statistically significant samples are hard to obtain and labour intensive to process.
- Vital to know absorbed DOSE.

Thanks to:

- James Murray, Robin Owen, Enrique Rudino-Pinera, LMB.
- Raimond Ravelli, EMBL
- Sean McSweeney, ESRF
- Martin Weik, IBS
- Dominique Bourgeois, IBS
- Graeme Laver, Murrambateman.
- Peter O'Neill, MRC, Harwell.
- ESRF Grenoble for LS2047 (and now MX-161)
- Royal Society Equipment Fund, EMBL, ESRF (microspec)

The Crystallographer's DILEMMA:

Damage onset versus diffraction intensity